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Experimental validation of RNA interference efficiency and off-target prediction in barley

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Faculty of mathematics, natural sciences, informatics

Bachelor thesis

Experimental validation of RNA interference efficiency and off-target prediction in barley

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Abstract:

This Bachelor thesis provides an experimental validation of the "si-Fi" software, which was designed for RNAi off-target searches and silencing efficiency predictions. The experimental approach is based on using synthetic DNA as RNAi-target as well as RNAi-trigger sequence. The data was generated by two different types of experiments using a transient gene silencing system in bombarded barley epidermal cells. The efficiency of RNAi was estimated by scoring the effect of silencing of the susceptibility-related gene *Mlo* on resistance of transformed cells to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* by observing reduction of fluorescent signals coming from an RNAi target fused to the green fluorescent protein.

The aim of this work was a comparison between *in silicio* prediction of RNAi efficiency and off-target effects in barley and experimental data.

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List of Abbreviations

ACS	1-aminocyclopropane-1-carboxylate synthase
AGT	appressorial germ tube
Amp	Ampilicin
APP	appressorium
ATP	Adenosine-5'-triphosphate
Bgh	*Blumeria graminis* f. sp. *hordei*
bp	base pairs
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
ESH	elongating secondary hyphae
EST	Expressed Sequence Tag
GFP	green fluorescent protein
GUS	ß-Glucuronidase
HAU	haustorium
hpi	hours post inoculation
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research
Kan	Kanamycin
Mlo	Mildew locus O
mRNA	Messenger RNA
nt	nucleotide
PAZ	Piwi Argonaut and Zwille
PGT	primary germ tube
RDRP	RNA-dependent-RNA-Polymerase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
shRNA	short hairpin RNA
SI	susceptibility index
siRNA	small interfering RNA

SM	SmartLadder
TIGS	Transient-induced gene-silencing
X - Gluc	5-Bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid

1 Introduction

1.1 RNA interference

RNA interference (RNAi) is a biological process where RNA molecules can suppress gene expression, typically by targeted destruction of specific mRNA molecules. This can be used to silence specific genes. RNAi is an ancient natural antiviral mechanism which has now been observed in many organisms such as plants, animals and fungi (Agrawal et al., 2003). The stepwise discovery of RNAi led to various names in the history of this mechanism including "co-suppression" (Napoli et al., 1990), "post-transcriptional gene silencing" (Ingelbrecht et al., 1994) and "quelling" (Cogoni et al., 1996). In 1998 Andrew Fire and Craig Mello published the technique of RNAi, in which double stranded RNA (dsRNA) in *Caenorhabditis elegans* led to an efficient and specific gene knockdown (Fire et al., 1998). For this Work Andrew Fire and Craig Mello shared the Nobel Prize in Physiology or Medicine in 2006. There exist several related RNAi pathways in animals, fungi and plants that differ in the type of double-stranded triggering RNA and in the involved multigene-family member of dicer, argonaute etc (Agrawal et al., 2003). A general and simplified view of the mechanism of RNA interference is shown in Figure 1.

The RNAi mechanism typically is triggered by double stranded RNA (dsRNA). Artificially, the formation of dsRNA can be achieved for example by introducing hairpin constructs or antisense constructs into the cell. In the next step the dsRNA is degraded to 19-25 bp fragments described as small interfering RNA (siRNA), by a ribonuclease enzyme in the RNase III family commonly called Dicer or Dicer-like (DCL). Dicer has four distinct domains: (1) an aminoterminal helicase domain, (2) dual RNase III motifs, (3) a dsRNA binding domain and (4) a PAZ domain named after the proteins Piwi Argonaut and Zwille (Agrawal et al., 2003; Kim, 2005). Divalent metal ions such as Mg^{2+} and Mn^{2+} are required as cofactors in the dicer protein (Blaszczyk et al., 2001). The RNase III domain of Dicer requires Adenosine-5'-triphosphate **(**ATP) (Elbashir et al., 2001).

Dicer together with other proteins as well as RNA forms the so-called RNA-induced silencing complex (RISC) (Hammond et al., 2001) that is capable of targeted degradation of specific mRNA and knockdown of gene expression.

RNAi can be a very efficient process because a few dsRNA molecules are sufficient to ensure a long period of the mRNA degradation. The reason for this phenomenon is that RISC can digest multiple mRNA molecules and a RNA-dependent-RNA-Polymerase (RDRP) uses the resulting fragments as primers for new RNA synthesis resulting in signal amplification (Lipardi et al., 2001).

Another known mechanism of gene silencing in plants is RNA-directed DNA methylation which often is called transcriptional gene silencing. This involves methylation of promoter regions or of histones and can be triggered by siRNA, viral or transgenic RNAs (Mette et al., 2000).

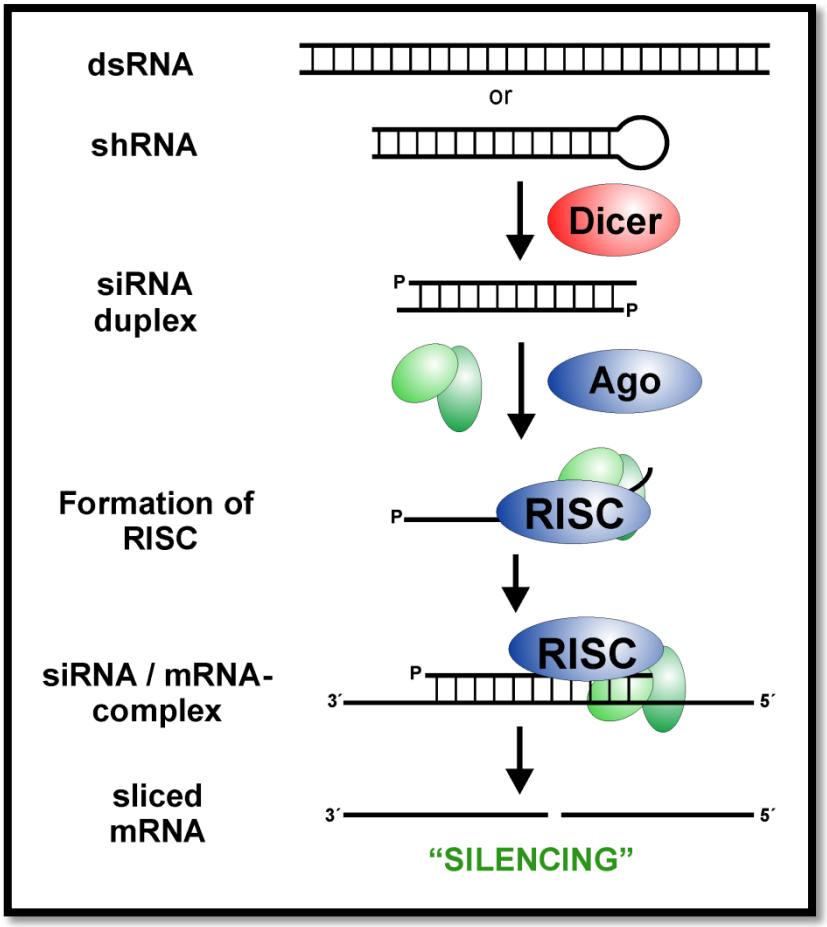

Figure 1: Simplified RNA interference mechanism [URL 1]
At first dsRNA or shRNA must be introduced into the cell. Then the enzyme Dicer cut the dsRNA or shRNA into siRNA. After that an Argonaute (Ago) protein and other proteins build the RNA-induced silencing complex (RISC). Finally RISC cuts the target mRNA and the gene will be silenced.

A major problem of RNAi when used as gene silencing technology is the so called off-target effect. This term describes the accidental silencing of genes in addition to the

target gene. If an siRNA population is partially homologous to an mRNA, transcribed from a gene that was not intended to be silenced, an off-target effect can occur. There are also several other possible factors during RNAi pathways which can trigger off-target silencing. The unwanted silencing can also be caused by RDRP-dependent, transitive silencing spreading out of the region covered by an RNA hairpin. Off-target effects have been observed in animals and plants and can be a risk for biosafety, because they can trigger unpredictable reactions in these organisms. Therefore it is important to check whether there is an off target or not in the organism to be studied or genetically modified (Senthil-kumar & Mysore, 2011).

1.2 The "si-Fi" software tool

Until now there are no versatile tools for defining parameters for RNAi efficiency and for off-target prediction in plants. Because of this, members of the working group Pathogen-Stress Genomics at IPK developed open-source software called "si-Fi" (siRNA-Finder).

The Software is a nucleotide sequence scanning tool, which can predict potential siRNA of 21 nucleotides length generated by a selected sequence if this sequence is introduced directly or indirectly as dsRNA into a plant cell. All of the predicted siRNAs are checked for occurrence of potential targets within a custom sequences database. The main goal of "si-Fi" is to optimize and customize dsRNA-generating transgenes and to predict off-targets of the selected RNAi-triggering sequences. The tool is designed to be applied for checking of long-dsRNAi constructs. It is not suitable for checking or designing, miRNA, amiRNA and so on.

Additionally the "si-Fi" algorithm can predict the silencing efficiency of the siRNAs based of rules defined and accepted by the user. The following default parameters of the "si-Fi" software are proposed: i) a G/C content between 35% and 60%, ii) at least 3 of the first 7 nucleotides at the 5`end of the antisense strand are A/U, and iii) the antisense strand must start with A/U. These default parameters for defining efficient siRNA are an educated guess due to studies on conditions and guidelines for mammalians (Gong et al., 2006; Naito et al., 2004, 2005; Pei & Tuschl, 2006; Ui-Tei et al., 2004), besides

studies for plants, which are also based on rules for mammalians (Xu et al., 2006). However, further adjustments to define efficient siRNA in plants have remained unsolved. This parameterization option gives the user control over the parameters for defining efficient siRNA [URL 2].

The graphical user interface can be seen in Figure 2.

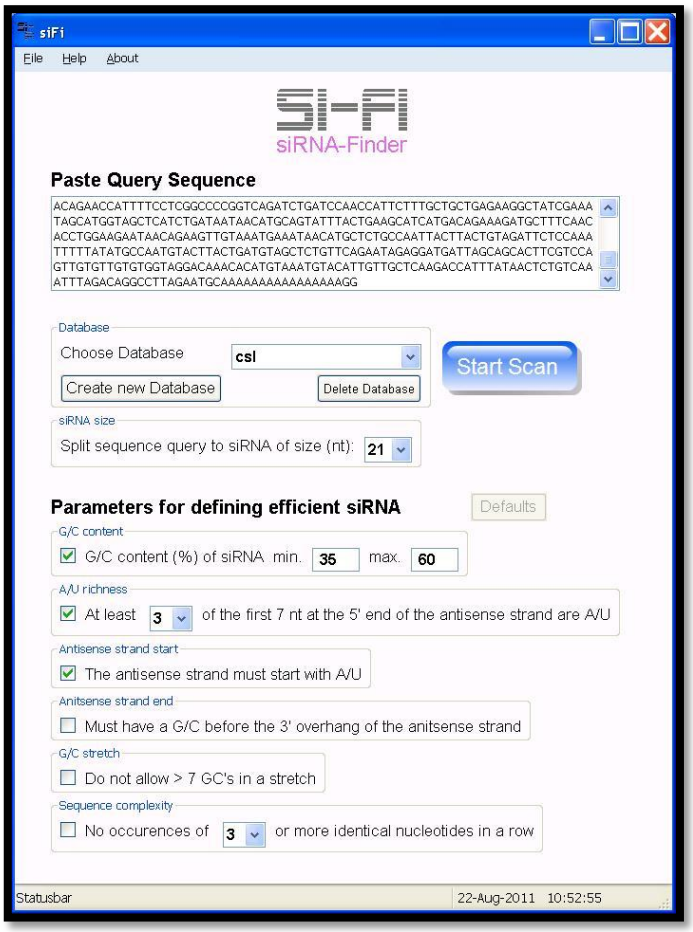

Figure 2: Graphical user interface "si-Fi" software [URL 2]
The query sequence is pasted in the sequence entry box and scanned against the selected database. Furthermore it is possible to change the siRNA size and the parameters for defining of efficient siRNA molecules.

The "si-Fi" software uses the open source *bowtie* algorithm. *Bowtie* is an ultrafast, memory-efficient short read aligner, which is developed towards quickly aligning large sets of short DNA sequences against large sequence databases (Langmead et al., 2009). Unlike other popular tools, such as BLAST (Madden, n.d.) *Bowtie* does not use a heuristic algorithm, and therefore it will find and report all possible hits. The "si-Fi" software functions as follows:

The process starts with creating a new sequence database by loading a FASTA file. After selecting a FASTA file, the *bowtie*-build indexer builds an index of the DNA sequences. The output is a set of files which together constitute the index. This index represents the new database and the original multiple FASTA file is no longer used [URL 9].

To start a scan with "si-Fi" an existing database must be selected and a query RNAi triggering sequence must be pasted into the corresponding field. With the start of the scan the query sequence will be split into siRNA with a size selected by the user. Both forward and reverse complement sequences will be created. After that the *bowtie*-aligner searches for siRNA matches against the selected database. The software will report all valid alignments per read. In the next step the "si-Fi" software takes the *bowtie*-aligner results and checks every siRNA hit, which was found, for efficiency. This check is according to the chosen parameters for defining efficient siRNA. Finally the software ends up with one list that includes all hits and another list that includes only the potentially efficient hits. For the graphical display, the software count how many siRNA nucleotides are at every query sequence position and plot all hits and the potentially efficient hits into one graphic.

1.3 Barley

Barley (*Hordeum vulgare* L.) is one of the most important crop plants in the world. It is a member of the tribe *Triticeae* within the grass family *Poceae* and belongs to the genus *Hordeum*. Cultivated barley (*Hordeum vulgare* spp *vulgare*) is a diploid organism with a large haploid genome of approximately 5.1 gigabases. It is one of the earliest domesticated crop plants in the world and represents the fourth most abundant cereal after wheat, maize and rice. As a crop species barley has particular importance because it is widely adapted to variable environmental conditions. In addition barley is more stress tolerant than wheat and because of this it remains a major food source in poorer countries. The range of uses implies mainly animal feed, human food and malt production. Beside its importance as a nutritional source barley has been established as a model organism, because it is a diploid, inbreeding and temperate crop. In addition large *ex-situ* germplasm collections are existing carrying huge number of potentially

valuable alleles. Enormous amount of expert knowledge concerning individual traits has been accumulated by the breeders. Valuable genomics resources were established for barley (The International Barley Genome Sequencing Consortium, 2012).

1.4 The phytopathogenic fungus *Blumera graminis*

Blumera graminis is an obligate biotrophic ascomycete belonging to the *Erysiphales*. It causes powdery mildew of grasses, including the crop plants wheat and barley. Cereals infected with *Blumera graminis* (B. *graminis*) have typical powdery "pustules" (Figure 3) produced by the mildew colonies and result into a reduced grain yield. *B. graminis* shows an extremely host specificity. The *"formae specialis" tritici* (wheat) and *hordei* (barley) can only infect the corresponding cereal species. All powdery mildews are strict obligate biotrophic pathogens, which means, that they are absolutely dependent on a living host plant (Dean et al., 2012).

Figure 3: Barley leaves infected with *B. graminis* f.sp *hordei* (Dean et al., 2012)
On the outer surface of the barley leaves grow the powdery mildew colonies.

1.5 Barley – *Blumeria graminis* - interaction

Powdery mildew epidemics are caused due to the short asexual life cycle of *B. graminis* and massive production of airborne spores (conidia). The 5 day cycle starts with the landing of conidia on barley leaf or stem surfaces. Within 0.5 hours post inoculation (hpi), a conidium germinates and a primary germ tube (PGT) emerges, which is dedicated to surface sensing. About 2 hpi the PGT is fully developed and approximately 5 to10 µm long. A few hours later an appressorial germ tube (AGT) is formed. The AGT is 30 to 40 µm long and separated from the conidium by a septum. A bat-like structure called appressorium (APP) is formed at the tip of the AGT. Approximately 10 - 12 hpi the APP forms a penetration peg which attempts to penetrate the plant cell wall. If this penetration is successful, the fungus develops its feeding organ called haustorium (HAU). The HAU invaginates but does not penetrate the cell membrane. Establishing of an HAU assures the further growth of the fungus and is a critical stage in fungal development. Approximately 24 hpi the fungus forms elongating secondary hyphae (ESH) which can differentiate into new AGTs forming secondary HAU. Four to five days post inoculation the fungal colony starts the production of conidiophores and the formation of ripe conidia. These can be dispersed easily by wind and let the cycle start again (Figure 4).

During its entire development *B. graminis* stays on the leaf surface and attacks only epidermal cells, which renders it readily accessible for microscopic studies (Figure 5). Because of this and other facts like: relatively easy to maintain, massive sporulation, fast and synchronous development, reduced biological complexity, easy to observe (surface growth), closely related "nonhost" *forma specialis* and high host specificity, sequenced genome and high agronomical relevance, *B.graminis* is a model organism to study other mildews and other obligate biotrophic pathogens (Baum et al., 2011; Both et al., 2005; Dean et al., 2012).

1.6 *mlo* resistance

Plant pathogen resistance can be introduced by mutations in single host genes. These mutants generally exhibit resistance to single or multiple classes of pathogens. The barley *mlo* gene mutant confers a broad spectrum resistance to *Blumera graminis* f.sp

hordei (Bgh). The cause for this is that the transmembrane MLO protein is a prerequisite for successful colonization by the powdery mildew fungus. In the absence of MLO, such as in barley *mlo* mutants, germinated fungal spores fail to enter epidermal host cells. The consequence is that the mutant plants are resistant. As a drawback the mutant plants exhibit spontaneous mesophyll cell death that appears to be part of accelerated leaf senescence (Devoto et al., 1999, 2003; Humphry et al., 2006; Piffanelli et al., 2002). Despite their disadvantages, the *mlo* resistance gene has been successfully used in barley cultivars for more than 30 years (Humphry et al., 2006).

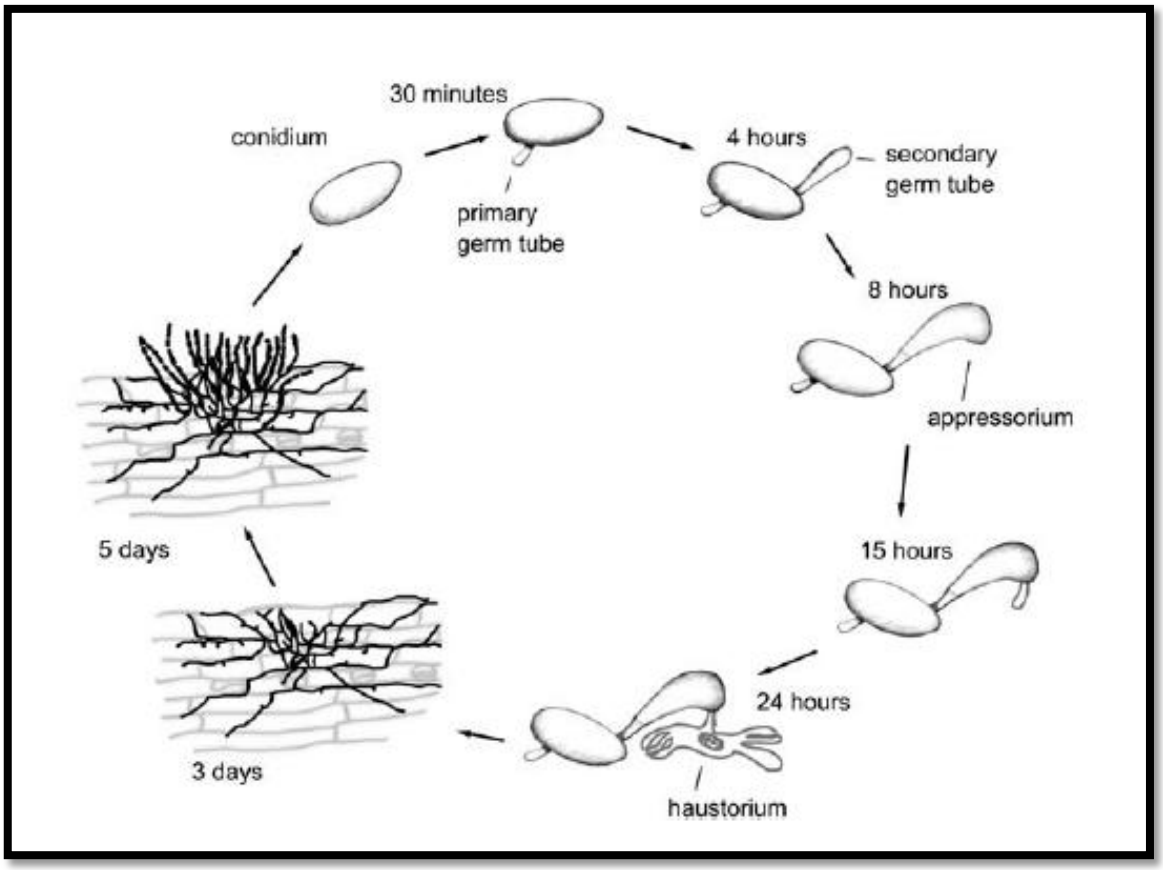

Figure 4: Asexual life cycle of *B. graminis* f.sp *hordei* (Both et al., 2005)
Post inoculation of barley the conidium form a primary germ tube (PGT) and then an appressorial germ tube (AGT), from which a peg penetrates though the host cuticle and epidermal cell wall. After the peg has successfully developed a haustorium (HAU) the fungus grows. Five days post inoculation the production of conidiospores starts, from which conidia can disperse and inoculate new barley plants. For further description see the text above.

Figure 5: *B. graminis* f.sp. *hordei* haustorium (Dean et al., 2012)
This figure shows the feeding organ haustorium (HAU) of *Blumera graminis* f.sp *hordei* (Bgh). Bar, 10 µm.

1.7. MLO family

The MLO gene family, including the first discovered barley MLO and homologues from other related plant species, is the only known family of seven-transmembrane proteins in plants. All MLO like proteins could be found within the genomes of several green land plants and this leads to that suggestion that they constitute a protein family. Their topology, subcellular localization, and sequence diversification are reminiscent of those of the G-protein coupled receptors from animals and fungi. *Mlo* like genes were identified in a broad range of plant species (Büschges et al., 1997; Devoto et al., 2003).
In completely sequenced genomes of model plants, like Arabidopsis *(Arabidopsis thaliana)* and rice (*Oryza sativa)*, a variety of MLO homologues have been identified. The MLO family comprises 437 entries (20 reviewed and 417 unreviewed) in the public sequence databases (Figure 6). From the reviewed UniProtKB entries are 15 from Arabidopsis, two from barley, two from rice and one from flax (The UniProt Consortium, 2013).

UniProt › UniProtKB — Downloads · Contact · Documentation/Help

Search | Blast | Align | Retrieve | ID Mapping *

Search in: Protein Knowledgebase (UniProtKB) — Query: (family:"MLO family") AND reviewed:yes — Search | Advanced Search » | Clear

20 results for family:"MLO family" AND reviewed:yes in UniProtKB sorted by organism ascending

Browse by taxonomy, keyword, gene ontology, enzyme class or pathway | Reduce sequence redundancy to 100%, 90% or 50% — Download

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Results Customize

› Add columns: Protein families

Entry	Protein names	Gene names	Organism	Length	Cross-reference (EMBL)
Q9FKY5	**MLO-like protein 10**	**MLO10** At5g65970 K2A18.3	Arabidopsis thaliana (Mouse-ear cress)	569	AF369571 mRNA. Translation: AAK53803.1. AB011474 Genomic DNA. Translation: BAB10402.1. CP002688 Genomic DNA. Translation: AED98133.1.
Q9FI00	**MLO-like protein 11**	**MLO11** At5g53760 MGN6.12	Arabidopsis thaliana (Mouse-ear cress)	573	AF369572 mRNA. Translation: AAK53804.1. AB017066 Genomic DNA. Translation: BAB09548.1. CP002688 Genomic DNA. Translation: AED96403.1. CP002688 Genomic DNA. Translation: AED96404.1. AY057502 mRNA. Translation: AAL09743.1. BT000434 mRNA. Translation: AAN17411.1. BT002581 mRNA. Translation: AAO00941.1.
O80961	**MLO-like protein 12**	**MLO12** At2g39200 T16B24.16	Arabidopsis thaliana (Mouse-ear cress)	576	AF369573 mRNA. Translation: AAK53805.1. AC004697 Genomic DNA. Translation: AAC28997.2. CP002685 Genomic DNA. Translation: AEC09645.1.
Q94KB2	**MLO-like protein 13**	**MLO13** At4g24250 T22A6.80	Arabidopsis thaliana (Mouse-ear cress)	478	AF369574 mRNA. Translation: AAK53806.1. AL078637 Genomic DNA. Translation: CAB45060.1. Sequence problems. AL161561 Genomic DNA. Translation: CAB79335.1. Sequence problems. CP002687 Genomic DNA. Translation: AEE84878.1.
Q94KB1	**MLO-like protein 14**	**MLO14** At1g26700 T24P13.8	Arabidopsis thaliana (Mouse-ear cress)	554	AF369575 mRNA. Translation: AAK53807.1. AC006535 Genomic DNA. Translation: AAF87028.1. Sequence problems. CP002684 Genomic DNA. Translation: AEE30722.1.
O80580	**MLO-like protein 15**	**MLO15** At2g44110 F6E13.24	Arabidopsis thaliana (Mouse-ear cress)	496	AF369576 mRNA. Translation: AAK53808.1. AC004005 Genomic DNA. Translation: AAC23431.1. CP002685 Genomic DNA. Translation: AEC10376.1.
O49621	**MLO-like protein 1**	**MLO1** MLO-H1 At4g02600 T10P11.12	Arabidopsis thaliana (Mouse-ear cress)	526	Z95352 mRNA. Translation: CAB08605.1. AC002330 Genomic DNA. Translation: AAC78258.1. AL161494 Genomic DNA. Translation: CAB80753.1. CP002687 Genomic DNA. Translation: AEE82202.1. CP002687 Genomic DNA. Translation: AEE82203.1. AY072135 mRNA. Translation: AAL59957.1. AY113992 mRNA. Translation: AAM45040.1.
Q9SXB6	**MLO-like protein 2**	**MLO2** At1g11310 T28P6.4	Arabidopsis thaliana (Mouse-ear cress)	573	AF369563 mRNA. Translation: AAK53795.1. AC007259 Genomic DNA. Translation: AAD49991.1. CP002684 Genomic DNA. Translation: AEE28715.1. AY086586 mRNA. Translation: AAM63648.1.
Q94KB9	**MLO-like protein 3**	**MLO3** At3g45290 F18N11.50	Arabidopsis thaliana (Mouse-ear cress)	508	AF369564 mRNA. Translation: AAK53796.1. AL132953 Genomic DNA. Translation: CAB72478.1. Sequence problems. CP002686 Genomic DNA. Translation: AEE78019.1.
O23693	**MLO-like protein 4**	**MLO4** At1g11000 T19D16.26	Arabidopsis thaliana (Mouse-ear cress)	573	AF369565 mRNA. Translation: AAK53797.1. U95973 Genomic DNA. Translation: AAB65495.1. Sequence problems. CP002684 Genomic DNA. Translation: AEE28674.1.
O22815	**MLO-like protein 5**	**MLO5** At2g33670 F4P9.44 T1B8.26	Arabidopsis thaliana (Mouse-ear cress)	501	AF369566 mRNA. Translation: AAK53798.1. AC002332 Genomic DNA. Translation: AAM14803.1. U78721 Genomic DNA. Translation: AAC69142.3. CP002685 Genomic DNA. Translation: AEC08867.1.
Q94KB7	**MLO-like protein 6**	**MLO6** At1g61560 T25B24.9	Arabidopsis thaliana (Mouse-ear cress)	583	AF369567 mRNA. Translation: AAK53799.2. AC005850 Genomic DNA. Translation: AAD25552.1. CP002684 Genomic DNA. Translation: AEE33851.1.
O22752	**MLO-like protein 7**	**MLO7** At2g17430 F5J6.19	Arabidopsis thaliana (Mouse-ear cress)	542	CP002685 Genomic DNA. Translation: AEC06624.1. AF369568 mRNA. Translation: AAK53800.1.
O22757	**MLO-like protein 8**	**MLO8** At2g17480 F5J6.21 MJB20.4	Arabidopsis thaliana (Mouse-ear cress)	593	AF369569 mRNA. Translation: AAK53801.1. AC007584 Genomic DNA. Translation: AAD32905.2. CP002685 Genomic DNA. Translation: AEC06633.1. BT002918 mRNA. Translation: AAO22734.1. BT004356 mRNA. Translation: AAO42350.1.
Q94KB4	**MLO-like protein 9**	**MLO9** At1g42560 F8D11.2 T8D8.5	Arabidopsis thaliana (Mouse-ear cress)	460	AC025815 Genomic DNA. Translation: AAG51314.1. Sequence problems. AC035249 Genomic DNA. Translation: AAG51234.1. Sequence problems. CP002684 Genomic DNA. Translation: AEE31927.1. AF369570 mRNA. Translation: AAK53802.1.
O49873	**MLO protein homolog 1**	**MLO-H1**	Hordeum vulgare (Barley)	544	Z95496 Genomic DNA. Translation: CAB08860.1.
P93766	**Protein MLO**	**MLO**	Hordeum vulgare (Barley)	533	Z83834 mRNA. Translation: CAB06083.1. Y14573 Genomic DNA. Translation: CAA74909.1.
P81785	**MLO-like protein**		Linum usitatissimum (Flax) (Linum humile)	217	AJ005341 mRNA. Translation: CAA06487.1.
A2YD22	**MLO protein homolog 1**	**MLO1** MLO-H1 OsI_022215	Oryza sativa subsp. indica (Rice)	540	Z95353 Genomic DNA. Translation: CAB08606.2. CM000131 Genomic DNA. No translation available.
Q0DC45	**MLO protein homolog 1**	**MLO1** MLO-H1 Os06g0486300 LOC_Os06g29110 OJ1568_D07.18 P0008F02.13	Oryza sativa subsp. japonica (Rice)	540	AP003518 Genomic DNA. Translation: BAD37345.1. Sequence problems. AP004012 Genomic DNA. Translation: BAD37627.1. Sequence problems. AP008212 Genomic DNA. Translation: BAF19578.1. Sequence problems. AF490386 Genomic DNA. Translation: AAO61753.1. Frameshift.

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Figure 6: Reviewed MLO family (The UniProt Consortium, 2013)
In the red rectangle the *Mlo* and *Mlo-H1* gene of barley are displayed, which were used in this work for the experimental validation. Note that the *Mlo-H1* has only a genomic DNA entry in GenBank and no expressed mRNA entry like the Mlo gene.

1.8 Particle bombardment / biolistics

A widely used method of transformation is the "biolistic particle bombardment", by instruments called "gene gun" or "biolistic particle delivery system". In that method the DNA is coated onto the surface of microscopic particles like gold or tungsten and then shot into cells, using a burst of helium gas. Microprojectile bombardment can transform such diverse targets as bacterial, fungal, insect, plant, animal cells and intracellular organelles. Both stable and transient transformations are possible with particle bombardment. This method was originally developed for plants and therefore whole plants can be regenerated from genetically modified cells by careful culturing and applying plant hormones (Bio-Rad Laboratories, n.d.; Halford & Shewry, 2000).
The bombardment process is explained in Figure 7.

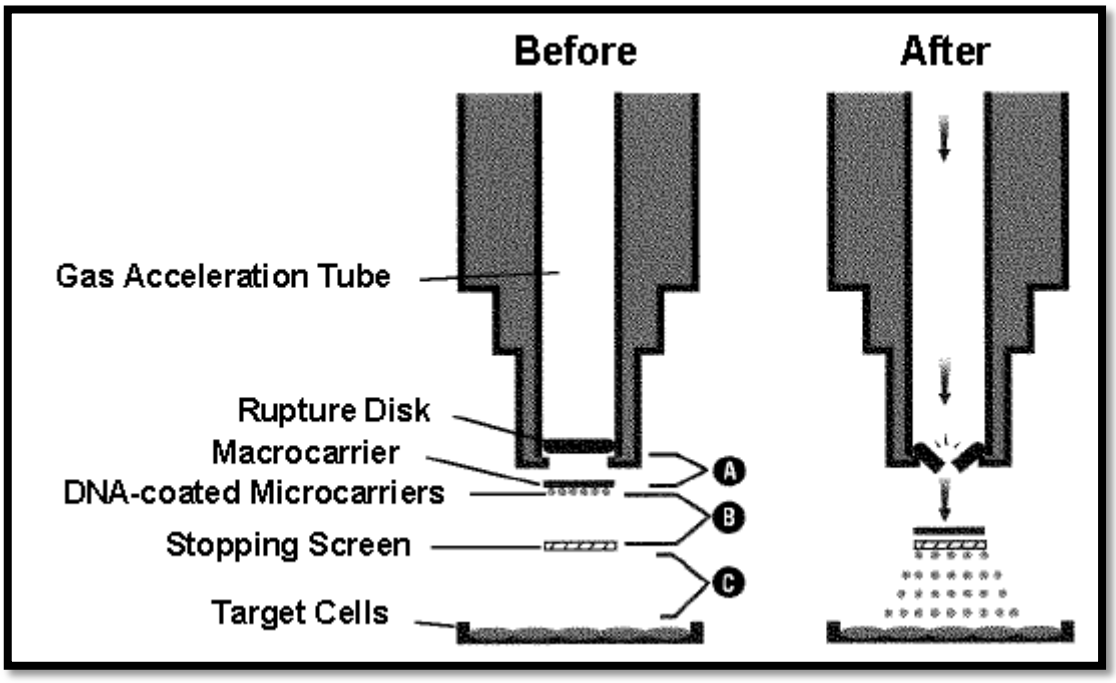

Figure 7: The biolistic bombardment process (Bio-Rad Laboratories, n.d.)
The biolistic system uses high pressure helium, released by rupture disk and partial vacuum to propel a macrocarriers sheet loaded with millions of microcarriers, for example with DNA coated gold particles. The macrocarrier is halted after a short distance by a stopping screen. The DNA coated microcarriers continuer traveling toward the target to penetrate and transform the cells. The launch velocity of microcarriers for each bombardment is dependent upon the helium pressure (rupture disk selection), the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the macrocarrier (Figure 7 A), the macrocarrier travel distance to the stopping screen (Figure 7 B), and the distance between the stopping screen and target cells (Figure 7 C). (Bio-Rad Laboratories, n.d.)

1.9 Aim

This Bachelor thesis should provide an experimental validation of the "si-Fi" software, which is designed for RNAi off-target search and silencing efficiency prediction. The approach of the work is based on using synthetic DNA as RNAi-target as well as RNAi-trigger sequence. The efficiency of RNAi will be estimated by scoring the effect of silencing of the susceptibility-related gene *Mlo* on the plant cell resistance to Bgh and by observing a reduction of the fluorescent signal coming from a RNAi target fused to green fluorescent protein (GFP). The aim of this work is a comparison between *in silicio* prediction of RNAi efficiency and off-target effects in barley with experimental data (Figure 8). The practical approach of the project is summarized in Figure 10.

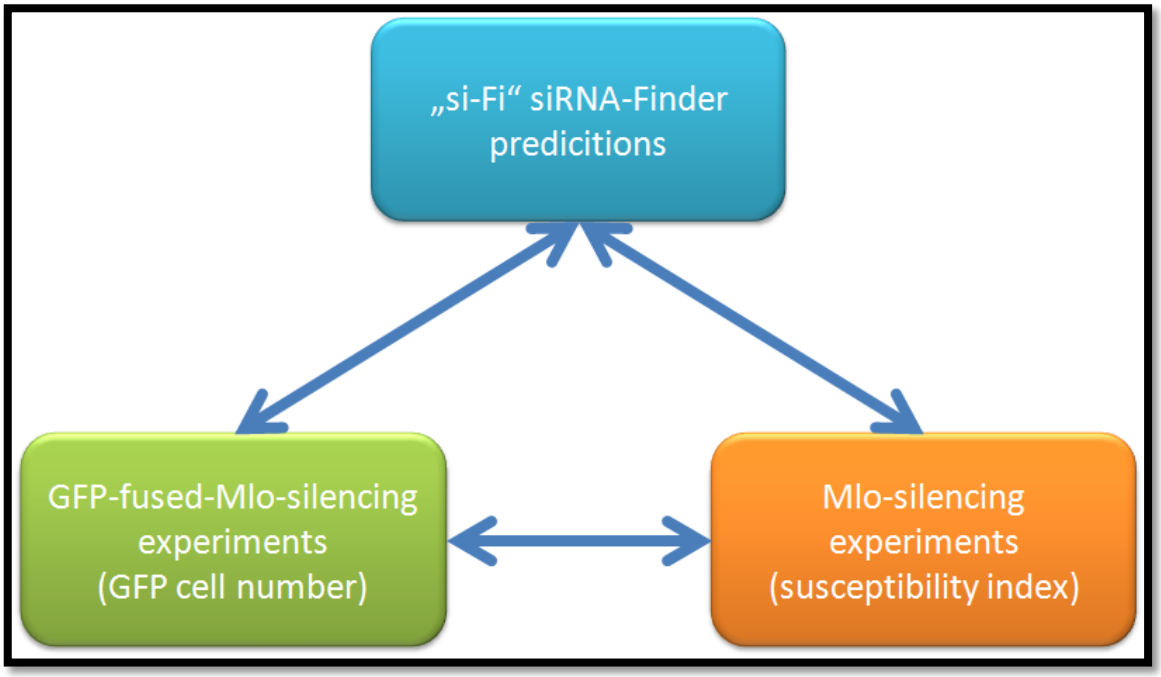

Figure 8: Comparison between the *in silico* prediction and the experimental data
This is a graphical design of the aim of this work. It displays a comparison between *in silicio* prediction of RNAi efficiency and off-target effects in barley with experimental data.

2 Materials and methods

2.1 Materials

2.1.1 Antibiotics

See Table 1 for an overview of the stock concentrations and the used final concentration of the used antibiotics. Ampilicin (Amp) was dissolved in 50% Ethanol/water (v/v) and Kanamycin (Kan) was dissolved in water.

Table 1: Overview of the used antibiotics

	stock concentration	final concentration
Ampilicin	100 mg/ml	1 g/ 10 ml
Kanamycin	50 mg/ml	0.5 g/ 10 ml

2.1.2 Enzymes and enzyme buffers

In Table 2 is an overview of the used enzymes and enzyme buffers. The restriction enzymes and related buffers were purchased from Thermo Scientific. The LR Clonase II Plus for Gateway cloning technology was purchased from Invitrogen. All enzymes were used according to manufacturer's instructions or as described in the respective sections.

Table 2: Overview of the used enzymes and enzyme buffers

	company	Lot number
***Xba*I**	Thermo Scientific	00125111
***Sal*I**	Thermo Scientific	00101712
T4 DNA Ligase	Thermo Scientific	00111328
***Eco*RV**	Thermo Scientific	00035051
T4 DNA Ligase Puffer	Thermo Scientific	00111328
Buffer Tango (10x)	Thermo Scientific	000020378
Buffer R (10X)	Thermo Scientific	00094895
Gateway® LR Clonase™ II Plus Enzyme Mix	Invitrogen	12538-200

2.1.3 Synthetic 500 bp *mlo* sequences

2.1.3.1 Generating of synthetic 500 bp *mlo* sequences

The used in this work 12 different synthetic 500 bp fragments were ordered at the company Genscript as pUC57 cloned inserts. The exact *Mlo* sequences from 0 to 100 % similarity as well as the MLO protein homolog 1 (*Mlo-H1*) sequence are shown in Appendix A. The 0 to 90% similar to *Mlo* sequences were generated by self-written Python tool (Appendix B for pseudocode) by replacing random nucleotides with dissimilar nucleotides to reaching the desired percent similarity. The 100% *Mlo* was taken from Genbank: Z83834.1 "*H. vulgare* mRNA for Mlo protein" and the homologues from GenBank: Z95496.1 "*H. vulgare Mlo-H1* gene".

2.1.3.2 ClustalW2 multiple sequence alignment

The multiple sequence alignment of the 12 synthetic sequences was performed with ClustalW2 [URL-10]. The following changes were made to the default settings: In "STEP 2 - Set your Pairwise Alignment Option" the GAP OPEN score was set to 100 and the GAP EXTENSION score to 10.0. In "STEP 3 - Set your Multiple Sequence Alignment Options" the GAP OPEN score was set to 100, the GAP EXTENSION score to 10.0 and the ORDER to Input.

2.1.4 Plasmid vectors and plasmid constructs

All used plasmid vectors in Table 3 and plasmid constructs in Table 4 were provided by Pathogen-Stress Genomics group at IPK.

Table 3: Overview of the used plasmid vectors

Vector	Short description	Reference
pIPKTA38	Entry vector for LR reaction	(Douchkov et al., 2005)
pIPKTA30N	Destination RNAi vector for LR reaction	(Douchkov et al., 2005)
pIPKTA44	GFP fusion vector (Figure 17)	unpublished

Table 4: Overview of the used plasmid constructs

Construct	Short description	Reference
pIPKTA36	Positive control for RNAi-mediated gene silencing of the *Mlo* gene, which results in resistance against *Bgh*	(Douchkov et al., 2005)
pIPKTA30N_SNAP34RNAi	Construct for RNAi-mediated gene silencing which should increase the cellular susceptibility against *Bgh*	(Douchkov et al., 2005)
pBC17	Internal control for bombardment efficiency, rise to anthocyanin	(Schweizer et al., 2000)
pUbiGUS	Internal control for bombardment efficiency, *ß-Glucuronidase* (GUS) gene under control of the maize Ubiquitin promoter	(Schweizer et al., 1999)

2.1.5 Bacterial strains

For the transformations a competent *E. coli:* DH10B strain was provided by Pathogen-Stress genomics group.

2.1.6 Kits

Table 5: Overview of the used kits

	company	Lot number
QIAprep® Spin Miniprep Kit (250	QIAGEN GmbH, Hilden	139314036 + 145616058
JetStar™2.0 Plasmid Midiprep Kit	Genomed GmbH, Löhne	314.01.08.07.2.3
QIAquick® Gel Extraction Kit (250)	QIAGEN GmbH, Hilden	127143885

2.1.7 Software used

2.1.7.1 DNASTAR Lasergene®

DNAStar Lasergene is a proprietary suite of software tools for molecular biology analyses [URL 3]. In this work the software SeqBuilder from DNASTAR Lasergene® Version 10.1.1 was used to create all vector maps.

2.1.7.2 GIMP

GIMP is an acronym for GNU Image Manipulation Program. It is a freely distributed program for such tasks as photo retouching, image composition and image authoring [URL 4]. GIMP Version: 2.8 were used to process all gel electrophoresis images. In all these images the colors were inverted and the relevant labels were placed.

2.1.7.3 LabTools

LabTools is a program for molecular biologists developed to assist them in common calculations in the lab, e.g. DNA ligation ratios, oligo annealing temperatures in a PCR reaction, solution calculator or unit conversions [URL 2]. For this work, the "ligation Calculator" of LabTools Version: 2.1.2-0008Beta was mainly used to calculate the required volumes for the ligation reactions.

2.1.7.4 “si-Fi” (siRNA Finder)

The prediction of targets and off-targets was done with the „si-Fi“ Version 3.1.0 (see section 1.2). All 12 *mlo* sequences were checked against the HarvEST:Barley Version 1.73 database, sequence assembly Nr. 35 [URL 11], with an efficient siRNA size of 21 nt and default parameters.

2.1.8 Chemicals

All chemicals were purchased from Carl Roth GmbH + Co. KG, Karlsruhe, except as otherwise stated.

In all gel electrophoresis the SmartLadder “200 to 10000 bp” from Eurogentec (Lot: 10D30-4) was used (Figure 9).

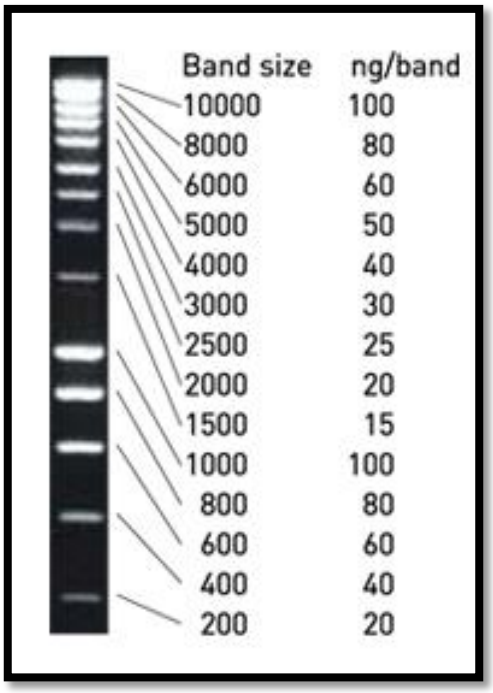

Figure 9: SmartLadder Eurogentec [URL 5]
The Figure shows the 14 regularly spaced bands from 200 to 10 000 bp. The 1000 and 10000 bp bands have a higher intensity than the others to allow quick and easy identification. The size of each band is an exact multiple of 100 bp [URL 5].

2.1.9 Generally used media and solutions

2.1.9.1 Media

SOC-media	
20 g/l	tryptone
5 g/l	Yeast extract
10 mM	NaCl
2.5 mM	KCl
autoclave	
Sterile filtering	
10 mM	$MgCl_2$
20 mM	glucose

LB-media	
10 g/l	tryptone
5 g/l	yeast extract
5 g/l	NaCl
pH 7,4	
1.5 % (w/v)	agar

2.1.9.2 Solutions

TAE	
40 mM	tris
20 mM	acetic-acid
1 mM	EDTA, pH 8.0

TE	
10 mM	tris-HCl, pH 8.0
1 mM	EDTA

2.1.10 Barley and growth conditions

In this work the barley cultivar "Golden Promise 2012" was used. The barley plants were grown in pots of compost soil from IPK. Approximately 100 - 150 cereal grains were put into them and covered with soil. Plants were grown in a climate chamber (SANYO, Versatile Environmental Test Chamber Model: MLR-350, Tokyo, Japan) at a temperature of 20°C, 16 h of light from fluorescent lamps, 8 h of dark and approximately 60 – 70 % relative humidity. They were poured with tap water.

2.1.11 *Blumeria graminis*

In this work *Blumeria graminis* f. sp. *hordei* (Bgh) (isolate 4.8 carrying *AvrMla*9) was used, which was provided by Pathogen-Stress genomics group. Bgh was maintained at 22°C and 16 h of light by weekly transfer to fresh barley cultivar Golden Promise.

2.2 Methods

2.2.1 General standard methods

2.2.1.1 Agarose gel electrophoresis

The separation of DNA to their fragment sizes were performed with agarose gel electrophoresis. For the gel electrophoresis a 1.0% Agarose Gel was made by solving 1% (w/v) agarose in 1x TAE buffer. The suspension was heated to complete melting of the agarose. Afterwards 1% (w/v) of the fluorescent dye Ethidium bromide (EtBr) was added after cooling down to about 55°C. Next the gel was poured into the gel electrophoresis tray with a corresponding gel comp, and was left to solidify for at least 30 minutes. The electrophoretic separation was carried out in 1x TAE buffer at 100V for about 45 minutes. Finally a picture of the gel was taken using the UV gel imaging system (INTAS Science Imaging Instruments GmbH, Göttingen, Germany).

2.2.1.2 Gel extraction with Qiagen: "QIAquick® Gel Extraction Kit"

The extraction of DNA fragments from agarose gels was performed as described in the kit protocol: "QIAquick Gel Extraction Kit Protocol using a microcentrifuge". The fragment were eluted from the column with 30 µL EB.

2.2.1.3 Plasmid isolation

The isolation of extrachromosomal plasmid DNA from bacteria was made with the "QIAprep® Spin Miniprep Kit" for minipreps or with the "JetStar™ 2.0 Plasmid Midiprep Kit" for midipreps. All steps were performed as described in the respective kit protocol. For both kits an overnight culture of bacteria was incubated under selection pressure of an antibiotic in LB medium at 37 ° C and 200 rpm agitation.

2.2.1.4 Optical density measurement

The quantification of isolated nucleic acids was performed by using a Photometer (Biophotometer, Eppendorf, Hamburg, Germany). A sample was diluted 1:100 and the concentration was determined. As reference "BLANK" sample, water was used.

2.2.1.5 Transformation of *E.coli*

To perform the transformations, 5 µl of the respective vector DNA were pipetted to competent *E.coli DH10B* cells. Then this mix was incubated for 30 minutes on ice. After that this mix was heat shocked at 42°C for 20 seconds. Subsequently it was chilled on ice for one minute. Thereafter 100 µl SOC media was added to the mix and it was incubated on a Thermomixer at 37°C and 800 rpm for one hour. At least 100 µl of each mix were spread on LB agar plates with an antibiotic as selection marker. These plates were incubated overnight at 37°C.

2.2.2 Creation of RNAi constructs

The pipeline of the creation of RNAi constructs and the experimental validation is summarized in Figure 10.

2.2.2.1 Ligation into entry vector

The twelve synthetic Mlo500 inserts in pUC57 cloning vector were digested with *Xba*I and *Sal*I. The *Mlo* fragments were isolated from an agarose gel as described in section 2.2.1.3. After this the *Mlo* fragments were ligated into a *Xba*I and *Sal*I digested pIPKTA38. The ligation reactions were performed for one hour at room temperature in 10 µl containing 1 µl of T4 DNA Ligase, 1 µl of T4 DNA Ligase buffer, 5 µl of the respective Mlo fragment (20 ng/µl) and 3 µl of the pIPKTA38 vector (65 ng/µl). The volumes of the components were calculated with the software "LabTools". The resulting pIPKTA38_MloRNAi(100-0%+H1) constructs were transformed as described in section 2.2.1.5 and spread on LB+ Kanamycin agar plates. One colony of each reaction was picked and inoculated for plasmid isolation as described in section 2.2.1.3. A control digestion of each clone was performed by restriction digestion with *Xba*I and *Sal*I. The positive pIPKTA38_MloRNAi(100-0%+H1) clones were used as donor vector in the LR reaction of the Gateway cloning system with pIPKTA30N.

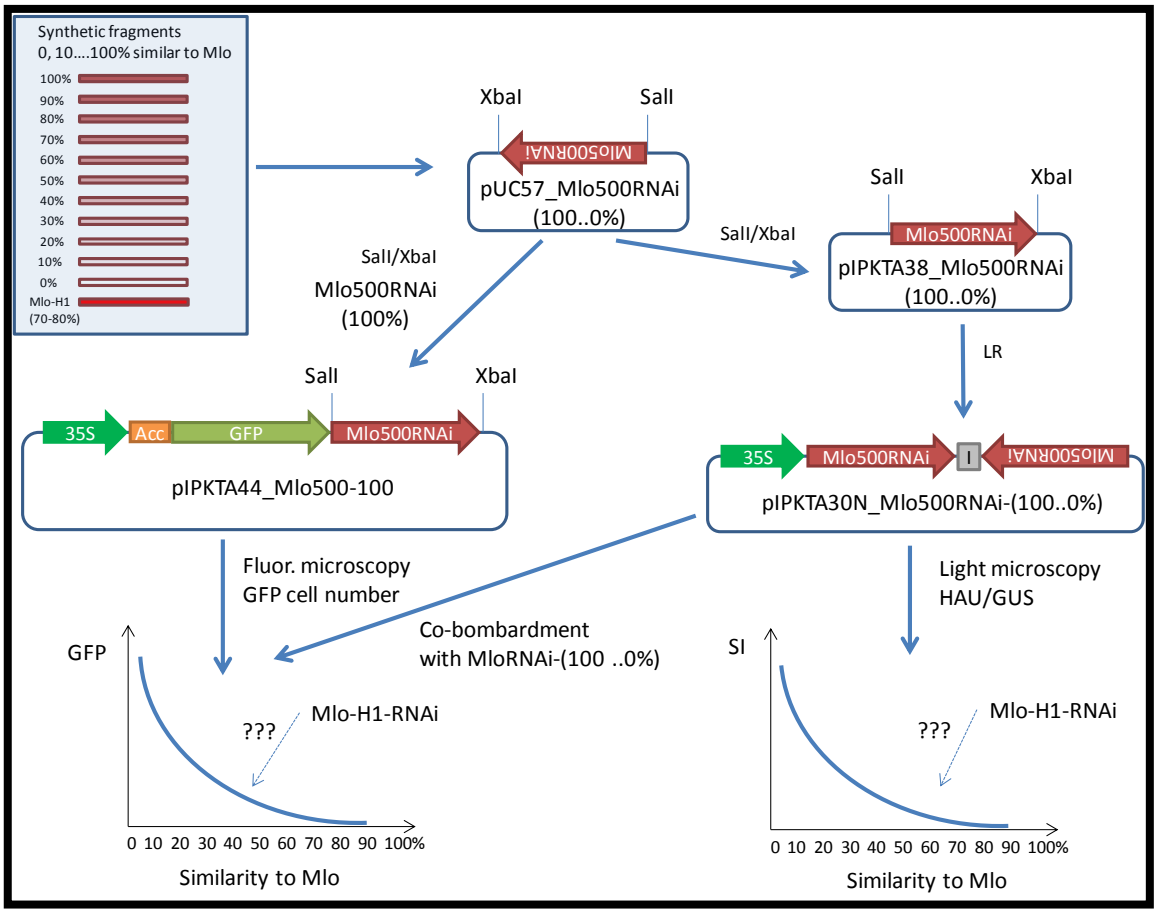

Figure 10: Pipeline for the experimental validation (D. Douchkov unpublished)
This Figure displays the experimental design for validation of RNAi efficiency and off-target prediction Software. The twelve synthetic fragments with similarities from 0 to 100% to the *Mlo* gene in addition with the *Mlo* homologues were cut out of delivery vector (pUC57) with the restriction enzymes *Xba*I and *Sal*I. The excised fragments were inserted to the Gateway™ entry vector pIPKTA38, and transferred by LR recombination reaction to the destination RNAi vector pIPKTA30N. In addition, the 100% similar to *Mlo* sequence will be transcriptional fused to a GFP reporter, and used as a target of the RNAi vectors created in the previous step. The silencing efficiency of the RNAi vectors was scored by means of reducing susceptibility of the cells to the barley powdery mildew (*B. graminis* f.s. *hordei* - Bgh). A 500 bp of 100% matching *Mlo* sequence will be cloned as 3' transcriptional fusion to GFP, and will be used as a target for the RNAi constructs.

2.2.2.2 LR reaction to destination vector

The *Mlo* fragments in pIPKTA38_MloRNAi(100-0%+H1) were cloned into the RNAi destination vector pIPKTA30N as inverted repeats by a single LR recombination reaction. The LR reactions were performed in a Volume of 10 µl containing 1.0 µl of the pIPKTA38_MloRNAi(100-0%+H1), 1.0 µl of pIPKTA30N destination vector (each 150 ng/µl), 2.0 µl of LR Clonase II mix and 6.0 µl H_2O. The reactions were incubated for 6 hours at room temperature. The resulting pIPKTA30NMloRNAi(100-0%+H1) constructs were transformed as described in section 2.2.1.5 and spread on LB+

Ampicilin agar plates. One colony of each reaction was picked and inoculated for plasmid isolation as described in section 2.2.1.3. At least a control digestion of each clone was performed by restriction digestion with *Eco*RV.

2.2.3 Creation of plasmid construct pIPKTA44_Mlo500_100

The synthetic Mlo500_100 insert in pUC57 cloning vector was digested with *Xba*I and *Sal*I. The *Mlo* fragment was isolated from an agarose gel as described in section 2.2.1.3. After this the *Mlo* fragment was ligated into a *Xba*I and *Sal*I digested pIPKTA44. The ligation reactions were performed for one hour at room temperature in 10 µl containing 1 µl of T4 DNA Ligase, 1 µl of T4 DNA Ligase buffer, 1.5 µl of the *Mlo* fragment (40 ng/µl), 2.5 µl of the pIPKTA44 vector (40 ng/µl) and 4 µl water. The volumes of the components were calculated with the software "LabTools". The resulting pIPKTA44_Mlo500_100 construct was transformed as described in section 2.2.1.5 and spread on LB+ Ampicilin agar plates. One colony of each reaction was picked and inoculated for plasmid isolation as described in section 2.2.1.3. At least a control digestion of each clone was performed by restriction digestion with *Xba*I and *Sal*I.

2.2.4 Transient expression by particle bombardment

2.2.4.1 Gold particle suspension

27.5 mg of gold powder (diameter 1µm, Bio-Rad, Hercules, USA) was added to 1 ml of sterile water. The suspension was mixed well and treated for 20 seconds in an ultrasonic bath. Then the gold was centrifuged at 14000 rpm for 30 seconds and the supernatant was removed. This whole wash procedure was repeated again with sterile water and then with 99.8% ethanol. Finally, the gold pellet was dried at 50°C for 10 minutes and dissolved in 1 ml 50% sterile glycerol by 30 seconds ultrasonication.

2.2.4.2 Coating of gold particles with DNA

Per shot 7 µl DNA (1 µg/µl) per construct and reporter construct were pipetted. The pipetting schemes of the experiments can be seen in Table 6 and Table 7. To each 87.5 µl ultrasonicated gold (27.5 mg/ml) suspension was added. While continuous mixing on a vortexer calcium nitrate $(CaNO_3)_2$ (pH = 10) was added drop wise to a final concentration of 0.5 M $(CaNO_3)_2$. These suspensions were incubated for approximately 10 minutes at room temperature by inverting from time to time. Then these suspensions

were centrifuged at 14000 rpm for 30 seconds and the supernatant were removed. After that the pellets were washed in 70% ethanol and then in 98.8% ethanol. At least the pellet was resuspendet in 30µl 98.8% ethanol.

Table 6: Pipetting scheme of the *Mlo*-silencing experiment
This table shows the pipetting scheme of the *Mlo*-silencing experiment. The empty pIPKTA30N RNAi vector was bombarded 3 times as internal control. pIPKTA36 is a positive control for RNAi-mediated gene silencing of the *Mlo* gene which should result in resistance against Bgh. pIPKTA30N_SNAP34RNAi is a control construct which should increase cellular susceptibility against Bgh. (Douchkov et al., 2005). For every shot was pUbiGUS containing the *ß-Glucuronidase* (GUS) gene under control of the maize Ubiquitin promoter as reporter gene for the transformed cells (Schweizer et al., 1999).

shot	Construct	µl Construct	µl pUbiGUS	µl Gold	µl Calcium nitrate
1	pIPKTA30N	7	7	87.5	101.5
2	pIPKTA30N_Mlo500RNAi-100	7	7	87.5	101.5
3	pIPKTA30N_Mlo500RNAi-90	7	7	87.5	101.5
4	pIPKTA30N_Mlo500RNAi-80	7	7	87.5	101.5
5	pIPKTA30N_Mlo500RNAi-70	7	7	87.5	101.5
6	pIPKTA30N_Mlo500RNAi-60	7	7	87.5	101.5
7	pIPKTA30N_Mlo500RNAi-50	7	7	87.5	101.5
8	pIPKTA30N_Mlo500RNAi-40	7	7	87.5	101.5
9	pIPKTA30N	7	7	87.5	101.5
10	pIPKTA30N_Mlo500RNAi-30	7	7	87.5	101.5
11	pIPKTA30N_Mlo500RNAi-20	7	7	87.5	101.5
12	pIPKTA30N_Mlo500RNAi-10	7	7	87.5	101.5
13	pIPKTA30N_Mlo500RNAi-0	7	7	87.5	101.5
14	pIPKTA30N_Mlo500RNAi-H1	7	7	87.5	101.5
15	pIPKTA36	7	7	87.5	101.5
16	pIPKTA30N_SNAP34RNAi	7	7	87.5	101.5
17	pIPKTA30N	7	7	87.5	101.5

Table 7: Pipetting scheme of the GFP-fused-*Mlo*-silencing experiments
This table shows the pipetting scheme of the GFP-fused-*Mlo*-silencing experiments. The empty pIPKTA30N vector bombarded 2 times per experiment as control. The RNAi constructs were co-bombarded with the pIPKTA44_Mlo500_100 construct and the pBC17 construct that gave rise to anthocyanin accumulation and served as internal control for bombardment efficiency in barley epidermal cells (Schweizer et al., 2000).

shot	Construct	µl Const-ruct	µl pBC17	µl pIPKTA44_Mlo500_100	µl Gold	µl Calcium nitrate
1	pIPKTA30N	7	7	7	87,5	108,5
2	pIPKTA30N_Mlo500 RNAi-100	7	7	7	87,5	108,5
3	pIPKTA30N_Mlo500 RNAi-90	7	7	7	87,5	108,5
4	pIPKTA30N_Mlo500 RNAi-80	7	7	7	87,5	108,5
5	pIPKTA30N_Mlo500 RNAi-70	7	7	7	87,5	108,5
6	pIPKTA30N_Mlo500 RNAi-60	7	7	7	87,5	108,5
7	pIPKTA30N_Mlo500 RNAi-50	7	7	7	87,5	108,5
8	pIPKTA30N_Mlo500 RNAi-40	7	7	7	87,5	108,5
9	pIPKTA30N_Mlo500 RNAi-30	7	7	7	87,5	108,5
10	pIPKTA30N_Mlo500 RNAi-20	7	7	7	87,5	108,5
11	pIPKTA30N_Mlo500 RNAi-10	7	7	7	87,5	108,5
12	pIPKTA30N_Mlo500 RNAi-0	7	7	7	87,5	108,5
13	pIPKTA30N_Mlo500 RNAi-H1	7	7	7	87,5	108,5
14	pIPKTA30N	7	7	7	87,5	108,5

2.2.4.3 Biolistic gene transfer

At first the 30 µl DNA-gold-suspension was distributed to the seven macro carriers (Bio-Rad), which were placed into the hepta adapter of a PDS-1000/He system (Bio-Rad, München, Germany). Primary leaf segments of seven-day-old barley seedlings were placed with the adaxial side up at a distance about 6 cm to the macro carriers onto 0.5% (w/v) phytoagar (Ducheva, Haarlem, Netherlands) in water containing benzimidazol at 20 ppm. Six leaves were used per bombardment. Then vacuum of 27.5 mmHg was made in the vacuum chamber. After that the leaves were bombarded at a helium pressure of 900 psi (900 psi rupture disk Bio-Rad) with the gold particles. At

least the leaves incubated in closed petri dishes at 20°C, 50% relative humidity, 16h light with an intensity of 12 µE/m^2/s and 8 h dark in a climate chamber (Panasonic, Versatile Environmental Test Chamber Model: MLR-352-H , Japan). The incubation time was depending on the experiment.

2.2.5 Inoculation with *Blumeria graminis*

Three days after bombardment, the barley leaves of the GUS shooting experiments were inoculated with Bgh. The inoculation density was approximately 150 – 200 conidia/mm^2. Afterwards the leaves were incubated again in the Panasonic climate chamber.

2.2.6 Staining of Mlo-silencing experiments

72 hpi with Bgh the X-Gluc staining were performed. The leaves were carefully collected with tweezers, superimposed to cut the leaves ends to the same length and transferred them into X-Gluc staining solution. The leaves were infiltrated 3 times with the X-Gluc staining solution in a vacuum exicator till the leaves go down to the bottom. After that they were incubated at 37°C for 24 h. To stop the staining reaction and increase the contrast, the X-Gluc staining solution was replaced by Trichloroacetic acid solution. The leaves were incubated for 10 minutes until complete decolorization of chlorophyll. Subsequently, they were washed twice with water and stored at 4 °C.

X – Gluc Solution	
0.1% (w/v)	5-Bromo-4-chloro-1*H*-indol-3-yl β-D-glucopyranosiduronic acid (X-Gluc)
20 % (v/v)	Methanol
0.1 M	NaH_2PO_4
0.1 M	Na_2HPO_4
10 mM	Na-EDTA
0.1 % (v/v)	Triton – X - 100
1.4 mM	Potassium ferrycyanide
1.4 mM	Potassium hexacyanoferrate (II)
pH	6.8 – 7.2

Trichloroacetic acid solution	
7.5 % (w/v)	Trichloroacetic acid
50 % (v/v)	Methanol

2.2.7 Microscopy

2.2.7.1 Microscopy of *Mlo*-silencing experiments

After staining of the *Mlo*-silencing experiments, the bombarded leaves were analyzed under the microscope (Axioskop, Carl Zeiss Microscopy GmbH, Germany) at magnification of 200 times by light microscopy. Scoring of interaction phenotypes was carried out by counting GUS-stained cells and the number of GUS-stained cells bearing at least one haustorium (Figure 11).

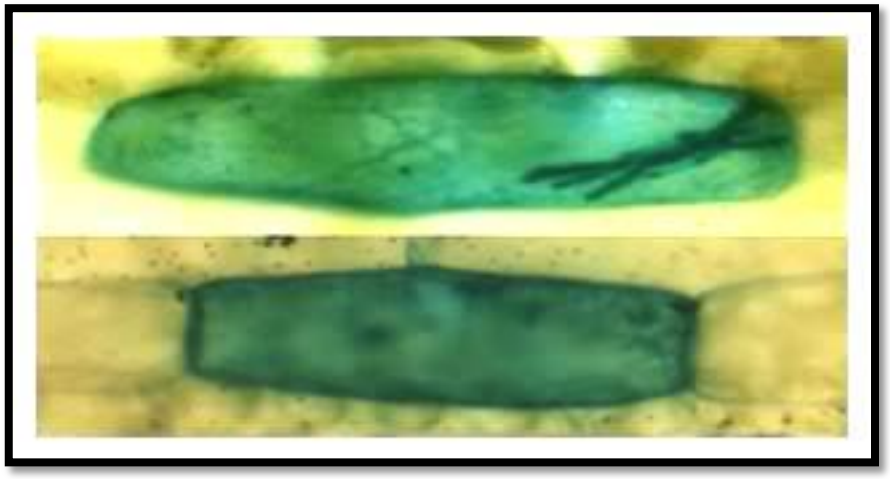

Figure 11: Transformed cells with expression of a GUS reporter gene [URL 6]
Haustorium formed successfully (top). No haustorium formed (bottom).

All GUS cells and GUS cells with haustoria of the six leaves per bombardment were added together and used to calculate the susceptibility index (SI).

$$susceptibility\ index\ (SI) = \frac{\Sigma\ GUS\ cells\ with\ Haustorium}{\Sigma\ GUS\ cells}$$

From all the shots with the empty vector of an experiment (pIPKTA30N), a mean value was calculated. The SI of the different constructs was determined and set in relation.

This is the relative SI as a percentage. This is considered to be a normalized measurement value of the sensitivity or rate of infection of the cells (Douchkov et al., 2005).

2.2.7.2 Microscopy of GFP-fused-Mlo-silencing experiments

At 24 h postbombardment the number of GFP expressing cells was counted by fluorescence microscopy (Axioplan 2 imaging, Carl Zeiss Microscopy GmbH, Germany). Data were normalized to the number of anthocyanin-accumulating cells 5 days postbombardment, which was counted by light microscopy.

$$normalized\ GFP = \frac{\Sigma\ GFP\ cells}{\Sigma\ anthocyanin\ accumulation\ cells}$$

From all the shots with the empty vector of an experiment (pIPKTA30N), a mean value was calculated. The normalized GFP of the different constructs were determined and set in relation. This is the relative normalized GFP as a percentage (Douchkov et al., 2005).

2.2.8 Statistical analysis of *Mlo*-silencing experiments

The statistical analysis, which is described in this section was based and performed on observations and rules of the Pathogen-Stress Genomics group.

At first all susceptibility indices, which are based on lesser than 50 GUS cells were removed. Then the 0% values were replaced with the default value of 13.0%, in order to prevent erratic values by log2 transformation. This corresponds to the average effect in approximately 150 experiments of the Pathogen-Stress Genomics group for the best resistance-inducing control pIPKTA36, which is targeting the Mlo gene. After that a Nalimov outlier test ($p < 0.01$) is applied on the values (percent) [URL 7].

$$q = \left|\frac{x_i - \bar{x}}{s}\right| \sqrt{\frac{n}{n-1}}$$

The resulting outliers are excluded in further calculations. In the next step all values were log2 transformed. At least a two-sided one sampled t-test [URL 8] is performed on the log2 values (Significant values: $p< 0.05$). This t test is performed against the

hypothetical relative susceptibility-index value "-0.355" corresponding to the observed median of more than 1000 RNAi constructs and is assumed to reflect a non-specific transient-induced gene-silencing (TIGS) effect. The reason for this type of calculation was the observation of the Pathogen-Stress genomics group that any RNAi construct has the tendency to induce some weak, sequence non-specific protection against *Blumeria graminis f.sp. hordei*, for reasons still unknown.

3 Results

Until now there are no common rules for defining parameters of RNAi efficiency and no experimentally validated versatile software tools for off-target prediction in plants. This asks for a combined *in silico* and experimental approach to that problem. The aim of the present Bachelor thesis was a comparison of *in silicio* prediction of RNAi efficiency and off-targets by the "si-Fi" software developed in the Pathogen-Stress Genomics group of the IPK and experimental data. The experimental assessment in plants of potential common rules as proposed in animal systems will remain a future task.

The experimental approach was based on synthetic DNA with different levels of similarity to the *Mlo* gene of barley. By incremental random mutagenesis of *Mlo* the desired percentage of similarity was reached. The *Mlo* gene is a negative regulator of cell death. Silencing of *Mlo* gene expression causes a strong increase of the resistance of transformed cells to powdery mildew fungi, which provides an easy observable and quantifiable phenotype.

A perfect match to the *Mlo* sequence (100% similarity) and 10 further sequences with decreasing similarity (from 90% to 0% in 10% steps approximately) where designed with a length of 500 bp. In addition a sequence derived from the MLO protein homolog *Mlo-H1* gene of barley sharing approximately 80% similarity to the *Mlo* gene was also included to the experiments as a natural gene potentially off-targeting *Mlo* (Appendix A).

With these previously designed sequences the *in silico* prediction was performed using the software "si-Fi", developed by the working group Pathogen-Stress Genomics at IPK.

For the experimental validation a transient gene silencing system in bombarded barley epidermal cells was used. In total twelve RNAi constructs including the synthetic sequences were generated. To perform this, these twelve synthetic fragments were cut out of the delivery vector (pUC57) with the restriction enzymes *Xba*I and *Sal*I. The excised fragments were inserted to the Gateway™ entry vector pIPKTA38, and transferred by LR recombination reaction to the destination RNAi vector pIPKTA30N.

The efficiency of the different RNAi constructs can be estimated by their effect on the resistance to Bgh. This was analyzed microscopically to determine the susceptibility index.

Another way to score the efficiency of the RNAi constructs was to fuse the original *Mlo* sequence transcriptionally to a GFP reporter. The resulting construct was used as a target of the RNAi constructs with decreasing similarity to *Mlo*. The silencing efficiency of the RNAi vectors was scored by means of determining reduction of the fluorescent signal of GFP.

3.1 Multiple sequence alignment

In Table 8 the percentage identity of the twelve synthetic sequences to *Mlo* can be seen. Random mutagenesis of 0-20% of nucleotide positions for sequences with 80-100% identity to *Mlo* generated sequences very close to the theoretical degree of mutagenesis. Random mutagenesis for 0-70% expected similarity resulted in less mutagenized sequences due to the heuristic alignment algorithm. The sequence of the *Mlo-H1* homologue has an identity to *Mlo* of approximately 80%.

Table 8: Percent identity matrix of *mlo* sequences
This table is the percent identity matrix of the 12 *in silico* designed *mlo* sequences. It was produced with ClustalW2 like described in section 2.1.3.2. The numbers in red display the percentage identities of the different similar *mlo* sequences in relation to the 100% *mlo* sequence. The black numbers show the percentage identity of each *mlo* sequence in comparison to another mlo sequence.

	mlo100	**mlo90**	**mlo80**	**mlo70**	**mlo60**	**mlo50**	**mlo40**	**mlo30**	**mlo20**	**mlo10**	**mlo0**	mloH1
mlo100	**100.00**	**90.20**	**80.40**	**74.80**	**67.60**	**60.40**	**66.00**	**48.60**	**46.80**	**42.20**	**34.20**	**80.69**
mlo90	**90.20**	100.00	73.60	68.80	61.00	55.00	60.80	45.60	43.80	38.80	31.20	71.80
mlo80	**80.40**	73.60	100.00	63.80	58.00	52.20	55.80	41.80	41.40	39.20	31.00	65.29
mlo70	**74.80**	68.80	63.80	100.00	53.00	48.80	52.20	39.40	39.20	37.80	29.80	63.56
mlo60	**67.60**	61.00	58.00	53.00	100.00	42.40	49.00	38.40	42.20	33.60	32.40	58.13
mlo50	**60.40**	55.00	52.20	48.80	42.40	100.00	44.20	35.00	36.40	31.60	30.20	53.15
mlo40	**66.00**	60.80	55.80	52.20	49.00	44.20	100.00	34.40	36.60	33.60	29.00	55.97
mlo30	**48.60**	45.60	41.80	39.40	38.40	35.00	34.40	100.00	30.60	30.80	28.40	42.30
mlo20	**46.80**	43.80	41.40	39.20	42.20	36.40	36.60	30.60	100.00	29.40	30.20	43.17
mlo10	**42.20**	38.80	39.20	37.80	33.60	31.60	33.60	30.80	29.40	100.00	30.00	38.61
mlo0	**34.20**	31.20	31.00	29.80	32.40	30.20	29.00	28.40	30.20	30.00	100.00	30.80
MloH1	**80.69**	71.80	65.29	63.56	58.13	53.15	55.97	42.30	43.17	38.61	30.80	100.00

3.2 "si-Fi" predictions

In Table 9 and Figure 12 the prediction of the „si-Fi" software is illustrated using HarvEST Barley assembly 35 cDNA sequence database [URL 11]. The prediction result suggests that a putative silencing effect of the selected RNAi-trigger sequences can be expected only for the constructs with 100% and partially with the 90% similarity as well as with the Mlo-homolog sequence. The maximum score of 390 total hits and 90 efficient hits are expected values for the 100%-construct, targeting the U35_16561 sequence, which represents barley mRNA of the MLO protein. For the 90% construct "si-Fi" still predicts 20 efficient siRNAs out of 39 total hits to the same U35 contig. For the remaining constructs the software could not find any putative siRNA hits.

For the RNAi construct based on the *Mlo-H1* the software predicted two efficient siRNAs out of 23 total hits to the U35_16561 contig. Only one hit to U35_24260, which is another sequence related to *Mlo* mRNA, was found.

Table 9: "si-Fi" predictions of *mlo* sequences against HarvEST assembly 35

Sequence	Id	All Hits	Efficient Hits
***mlo*_100**	U35_16561	390	90
***mlo*_90**	U35_16561	39	20
Mlo-H1	U35_16561	23	2
Mlo-H1	U35_24260	1	0
***mlo*_80 – *mlo*_0**	No hits found		

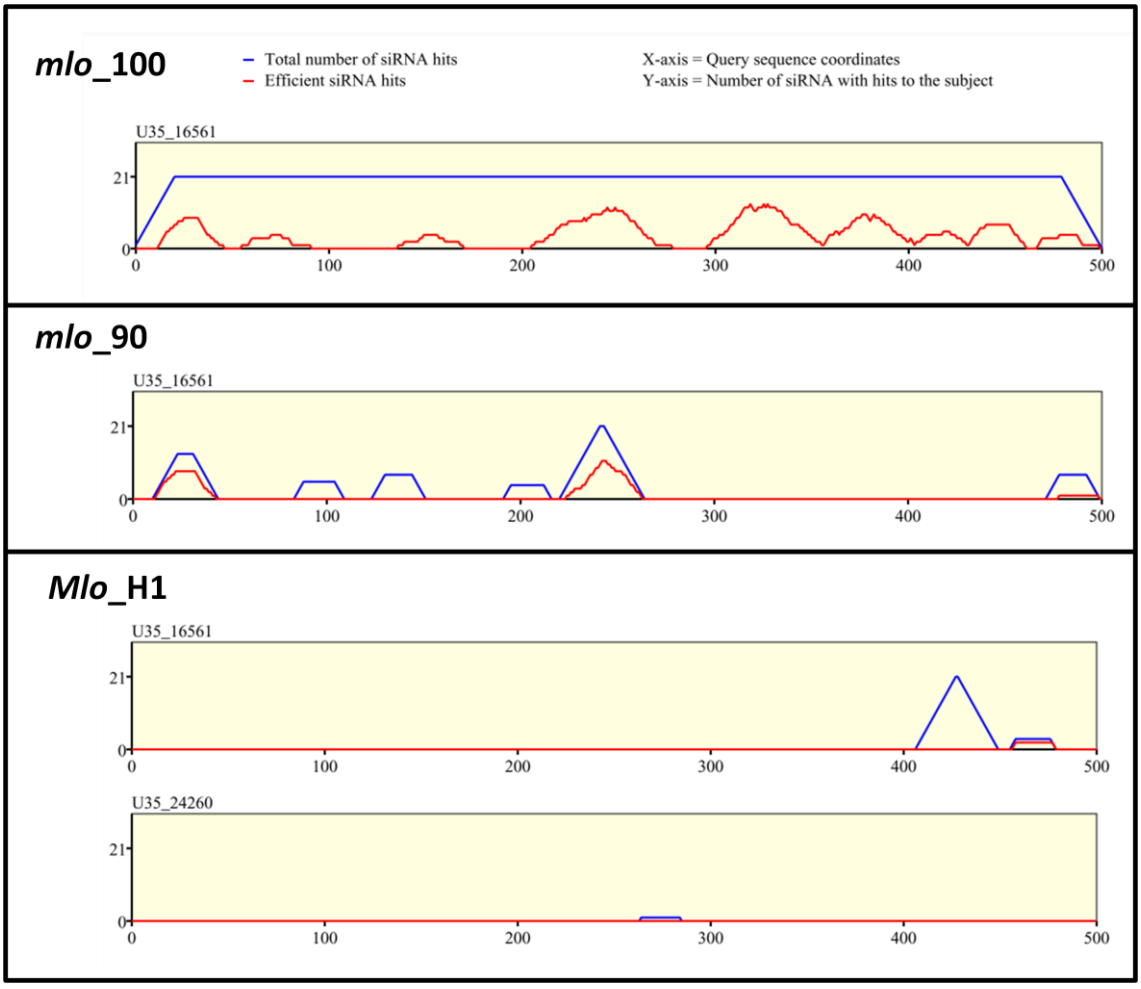

Figure 12: Graphical view of "si-Fi" predictions
This Figure shows the graphical output of the "si-Fi" software for the predictions of the mlo_100, mlo_90 and the *Mlo-H1* sequence against the HarvEST:Barley Version: 1.73 database.

3.3 Constructs

3.3.1 Plasmid constructs pIPKTA38_Mlo500RNAi(0-100%+H1)

All pIPKTA38 clones were analyzed by restriction enzyme digestion and gel electrophoresis as shown for an example in Figure 13. In the resulting twelve pIPKTA38_Mlo500RNAi(0-100%+H1) contructs the *Mlo* fragments are flanked by *attL*1 and an *attL*2 recombination sites and can be transferred to destination vectors in a Gateway LR recombination reaction (Figure 14).

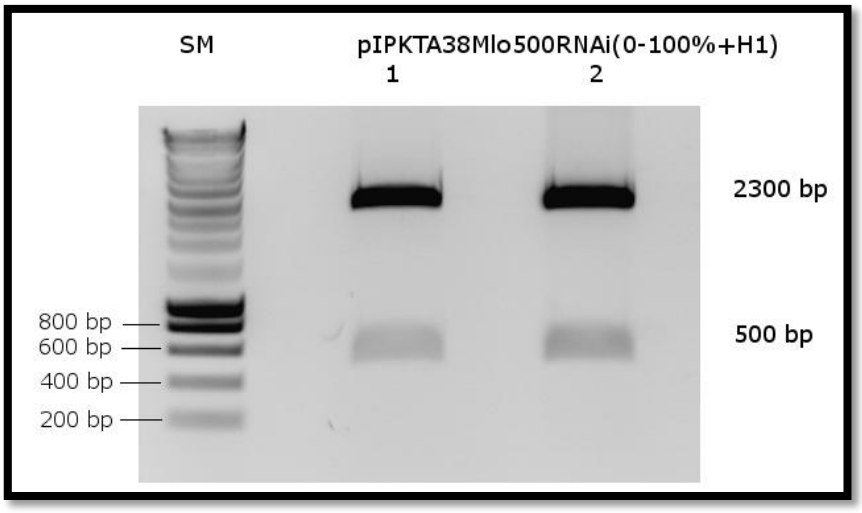

Figure 13: Control digestion of pIPKTA38Mlo500RNAi(0-100%+H1) with SalI and XbaI
The Figure shows on the leftmost the SmartLadder (SM) and two examples for the control digested pIPKTA38Mlo500RNAi(0-100%+H1) vector (with *Sal*I and *Xba*I) aside. There is clearly to see a band at 2300 bp (linearized vector) and at 500 bp (*Mlo* fragment).

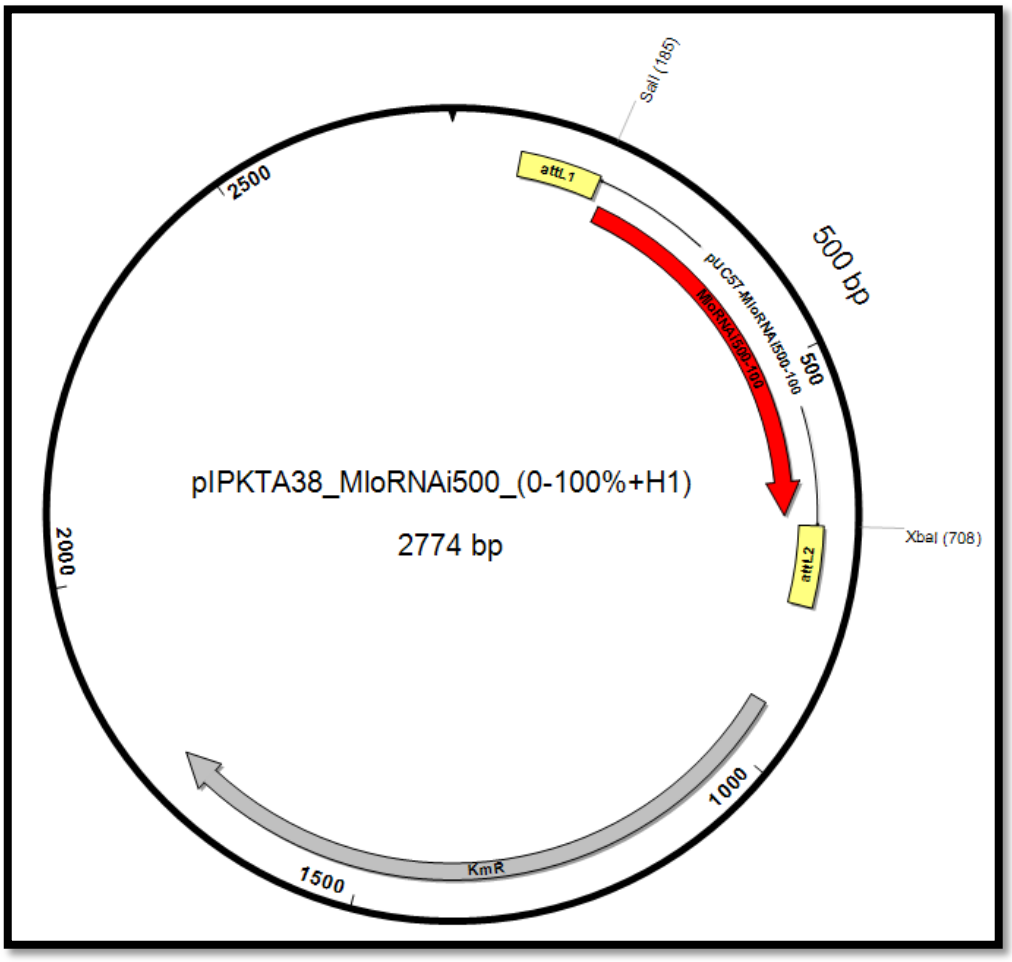

Figure 14: Plasmid vector pIPKTA38_Mlo500RNAi(0-100%+H1)
This is the donor vector in the LR reaction for the Gateway Cloning System. Between the restriction sites from SalI and XbaI and between the attl1 and attl2 recombination sites (yellow) is the Mlo fragment (red) of 500 bp. There is also a Kanamycin resistance gene (KmR (grey)) present, as selective marker.

3.3.2 Plasmid vector pIPKTA30NMlo500RNAi(0-100%+H1)

The *Mlo* fragments of pIPKTA38MloRNAi(0-100%+H1) were transferred into the destination Vector pIPKTA30N by Gateway LR recombination reaction. Figure 15 shows agarose gel analysis of the obtained pIPKTA30N clones digested by *Eco*RV. Each of the twelve resulting constructs carries two inverted repeats of the *Mlo* fragments separated by an intron (the second intron of the wheat RGA2 gene). The constructs contain the promoter and terminator sequences of 35S RNA of Cauliflower mosaic virus, as well as beta-lactamase gene for ampicillin resistance (Figure 16)

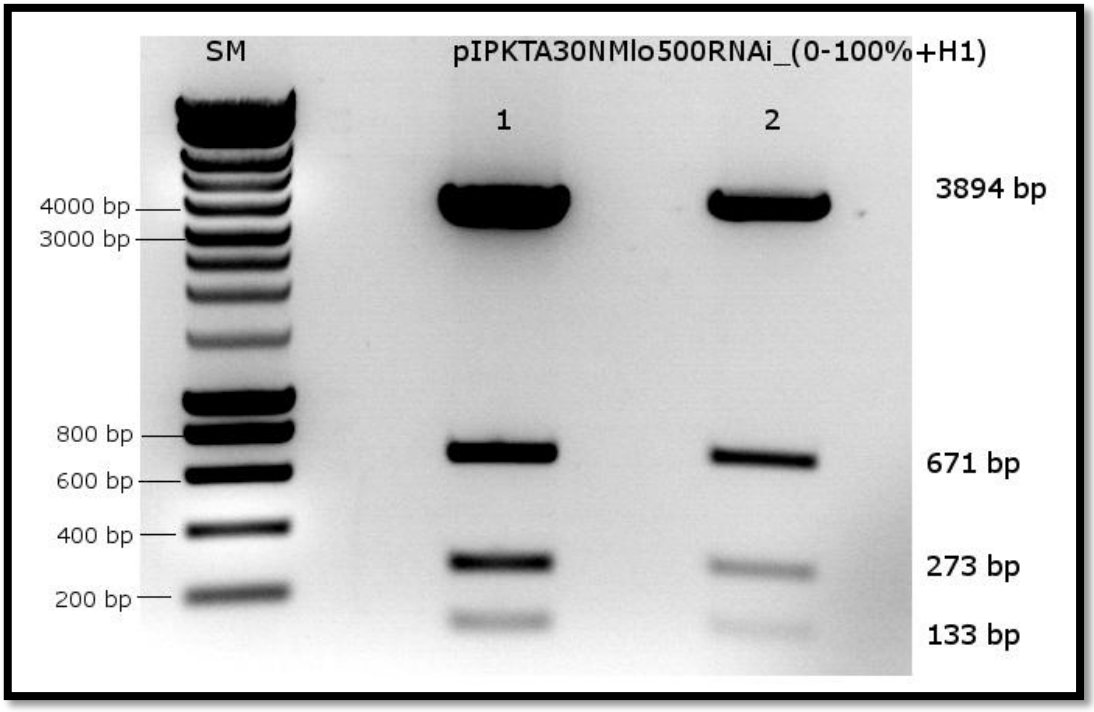

Figure 15: Control digestion of pIPKTA30NMlo500RNAi(0-100%) with EcoRV
The Figure shows on the leftmost the SmartLadder (SM) and two examples for the with EcoRV digested pIPKTA30NMlo500RNAi(0-100%+H1) vector aside. The bands of 3894 bp, 671 bp, 273 bp and 133 bp correspond to the expected fragment sizes, which are shown in Figure 16.

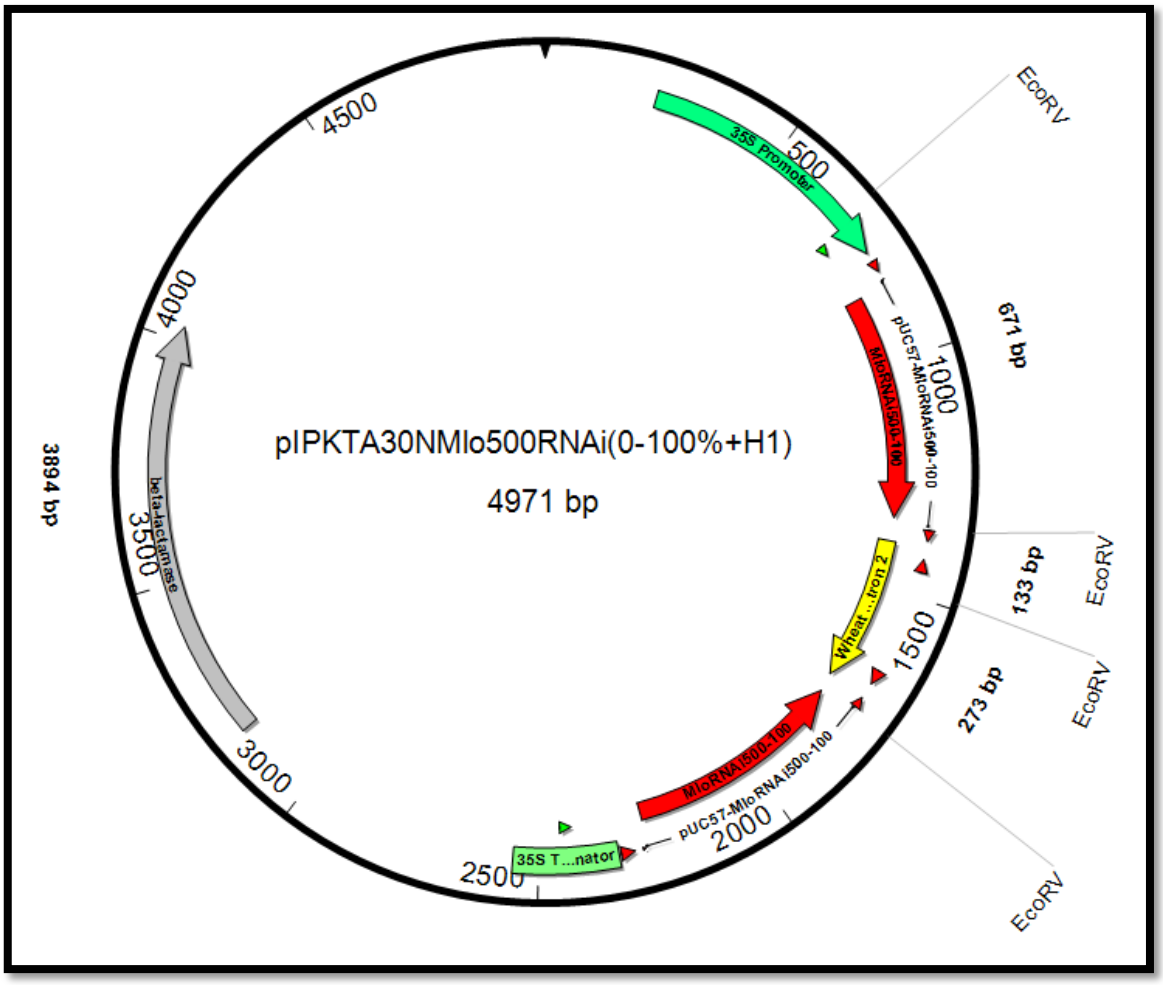

Figure 16: Plasmid vector pIPKTA30NMlo500RNAi(0-100%+H1)
The resulting pIPKTA30NMlo500RNAi(0..100%) construct after the LR recombination reaction. Between the inverted *Mlo* fragments repeats (red) is the second intron of the wheat RGA2 gene (yellow). The promoter and terminator (green) derived by the 35S RNA gene of CaMV35S of Cauliflower mosaic virus. There is also a beta-lactamase gene (grey) for selective medium. The restrictions sites for *Eco*RV and the resulting fragment sizes for the control digestion are shown.

3.3.3 Plasmid construct pIPKTA44_Mlo500_100

The pIPKTA44 clones were analyzed by by restriction enzyme digestion and gel electrophoresis. The resulting pIPKTA44_mlo500_100 is the GFP-fused RNAi-target construct of the GFP-fused–*Mlo*-silencing experiments. The promoter and terminator of the 35S RNA gene of *Cauliflower mosaic virus* (CaMV35S) drive expression of a fusion cassette consisting of: i) N-terminal part of a putative barley 1-aminocyclopropane-1-carboxylate synthase (ACS) causing instability of the GFP protein; ii) transcriptionally fused GFP open reading frame; iii) transcriptionally fused 500 bp barley *Mlo* sequence (100% similarity) as RNAi target (Figure 17). Due to the

ACS destabilization domain fusion proteins first fluoresced brightly but became no detectable at approximately three days after bombardment (Dong et al., 2006).

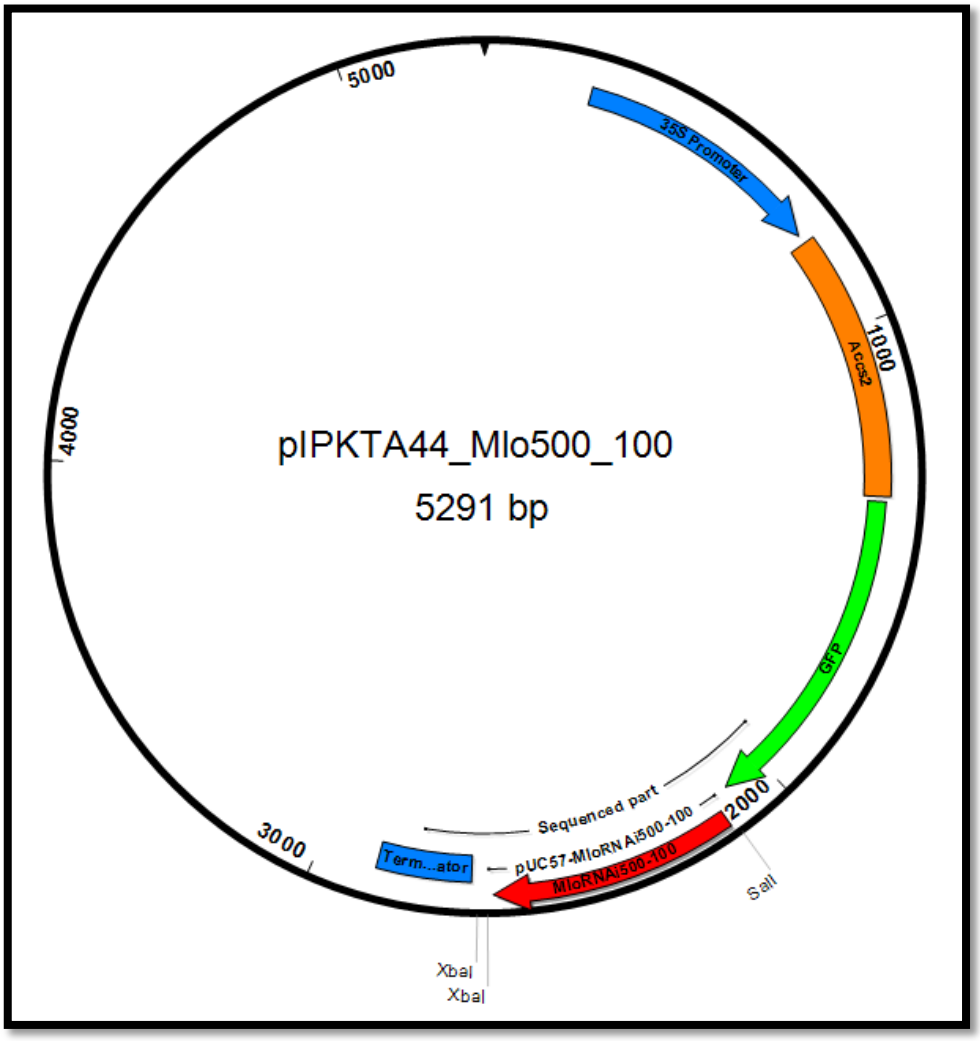

Figure 17: Plasmid vector pIPKTA44_Mlo500_100
The pIPKTA44_Mlo500_100 has a fusion cassette consisting of i) N-terminal part of a putative barley 1-aminocyclopropane-1-carboxylate synthase (ACS (orange)) causing instability of the protein; ii) transcriptionally fused GFP open reading frame(green); iii) 500 bp barley *Mlo* derived sequence (100% similarity (red)) as RNAi target. The promoter and terminator (green) derived by the 35S RNA gene of CaMV35S of Cauliflower mosaic virus.

3.4 *Mlo*-silencing experiments

The efficiency of the twelve designed and cloned different RNAi constructs was estimated by their effect on the resistance to Bgh. Therefore a TIGS system with the biolistic PDS-1000/He system (Bio-Rad, München, Germany) was used. Primary leaf segments of seven-day-old barley seedlings were co-bombarded with a corresponding RNAi construct and the pUbiGUS construct containing the *ß-Glucuronidase* (GUS)

gene under control of the maize Ubiquitin promoter as a reporter gene for the transformed cells (Schweizer et al., 1999). The empty pIPKTA30N RNAi vector was bombarded three times in each experiment as internal control. Furthermore a pIPKTA36 construct as positive control for RNAi-mediated gene silencing of the *Mlo* gene, which should result in resistance against Bgh and the pIPKTA30N_SNAP34RNAi construct which should increase cellular susceptibility against Bgh, were used. (Douchkov et al., 2005). The pipetting scheme for the *Mlo*-silencing experiments can be seen in Table 6.

Three days after bombardment the leaves were inoculated with Bgh and stained 72 hpi with X-Gluc solution and distained with TCA solution 24 hours after X-Gluc. The leaves were analyzed by light microscopy. Counting GUS-stained cells and the number of GUS-stained cells bearing at least one haustorium leads to the susceptibility index (SI). These SI were normalized to the empty pIPKTA30N RNAi vector per experiment. Data based on less than 50 counted GUS cells per shooting were removed, resulting in five to seven independent experiments for the *Mlo*-silencing experiments. Finally a statistical analysis of the data was done. The results can be seen in Table 10 and Figure 18. The control constructs produced the effect as expected and described in (Douchkov et al., 2005).

The standard *Mlo*-silencing pIPKTA36 construct induced a strong resistance to Bgh as expected. The pIPKTA30N_SNAP34RNAi construct showed a clear susceptibility-enhancing effect to Bgh also according to the prediction. This was also confirmed as statistically significant using a two sided t-test for both constructs. Furthermore the pIPKTA30N_Mlo500RNAi_100 construct with the 100% similarity sequence and the pIPKTA30N_Mlo500RNAi-H1 construct with the *Mlo-H1* sequence showed a resistance-enhancing effect similar to those of the positive control (pIPKTA36). The effect of the 100% similarity pIPKTA30N_Mlo500RNAi_100 construct was significant in two sided t-test and the effect from the homologue pIPKTA30N_Mlo500RNAi-H1 construct was significant in one-sided t-test. Upon mutagenesis the RNAi effect decreased very rapidly. Thus the designed 90% similarity pIPKTA30N_Mlo500RNAi_90 construct with just 10% less similarity in comparison to the 100% similarity pIPKTA30N_Mlo500RNAi_100 construct produced only a very small, non significant effect. The other designed RNAi constructs with a lower

similarity had no silencing effect. Unexpectedly, the statistical analysis also revealed significantly enhanced susceptibility of the construct with 20% sequence similarity to *Mlo*. However, for this pIPKTA30N_Mlo500RNAi_20 construct the calculated mean relative SI was 0.003 and therefore, only significant by assuming the non-specific TIGS effect as discussed above. Finally, we cannot exclude an off-target effect against an unknown gene that escaped "si-Fi" prediction.

In summary, only the 100% similarity pIPKTA30N_Mlo500RNAi_100 construct and the *Mlo-H1* targeting pIPKTA30N_Mlo500RNAi-H1 construct had a significant effect in these Mlo-silencing experiments. Supplementary data can be seen in Appendix C to Appendix H.

Table 10: *Mlo*-silencing experiments statistics
This Table shows the statistic of the results of the *Mlo*-silencing experiments. The data is based on the log2 transformation of SI. STD = standard deviation, SEM = standard error of mean, n = number of experiments, the green marked t-tests are significant ($\alpha < 0.05$)

Vector	Mean	STD	SEM	T-test (two-sided)	T-test (one-sided)	n
pIPKTA30N_Mlo500RNAi-100	-1.339	0.837	0.342	0.035	0.017	6
pIPKTA30N_Mlo500RNAi-90	-0.604	0.901	0.403	0.570	0.285	5
pIPKTA30N_Mlo500RNAi-80	-0.273	0.482	0.182	0.667	0.334	7
pIPKTA30N_Mlo500RNAi-70	-0.177	0.326	0.133	0.238	0.119	7
pIPKTA30N_Mlo500RNAi-60	-0.444	0.743	0.303	0.781	0.391	6
pIPKTA30N_Mlo500RNAi-50	-0.072	0.393	0.160	0.138	0.069	6
pIPKTA30N_Mlo500RNAi-40	-0.575	0.878	0.358	0.566	0.283	6
pIPKTA30N_Mlo500RNAi-30	-0.120	0.554	0.248	0.397	0.198	5
pIPKTA30N_Mlo500RNAi-20	0.003	0.452	0.171	0.081	0.040	7
pIPKTA30N_Mlo500RNAi-10	-0.258	0.413	0.169	0.589	0.294	6
pIPKTA30N_Mlo500RNAi-0	-0.379	0.436	0.165	0.889	0.445	7
pIPKTA30N_Mlo500RNAi-H1	-1.473	1.217	0.497	0.074	0.037	6
pIPKTA36	-1.745	0.844	0.319	0.005	0.002	7
pIPKTA30N_SNAP34RNAi	1.002	0.587	0.239	0.002	0.001	6

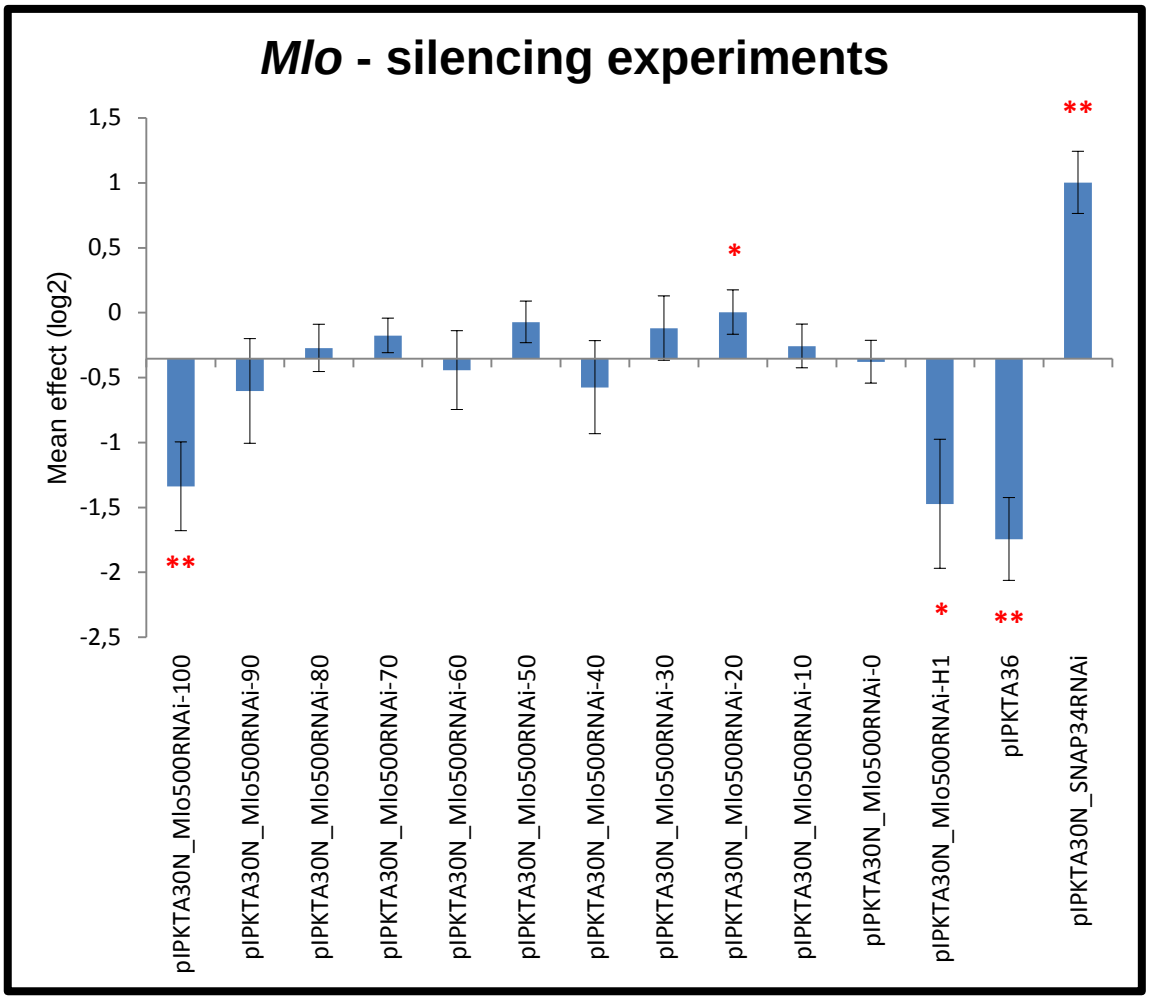

Figure 18: Statistics of *Mlo*-silencing experiments
The blue bars in this Figure show the mean effect (log2) of the Mlo-silencing experiment. The black bars shows standard error of means. Bars marked with ** show two sided significant effects and bars with * show one sided significant effects.

3.5 GFP-fused-*Mlo*-silencing experiments

Another way to examine the effectiveness of the different RNAi constructs was attempted in this Bachelor thesis. The approach was based on transcriptionally fusing the 100% similarity *Mlo* sequence as RNAi-target to the reporter gene GFP. After that the fusion target and the RNAi constructs were co-bombarded and the GFP signals were counted. In these GFP-fused-*Mlo*-silencing experiments the silencing efficiency of the twelve different RNAi vectors were scored by means of reducing the fluorescent signal of GFP.

For this experiment, the same TIGS system with the biolistic PDS-1000/He system (Bio-Rad, München, Germany) was used. Primary leaf segments of seven-day-old barley seedlings were co-bombarded with i) the new GFP construct

(pIPKTA44_Mlo500_100), ii) a corresponding RNAi construct and iii) the pBC17 construct that gave rise to anthocyanin accumulation and serves as internal control for bombardment efficiency in barley epidermal cells (Schweizer et al., 2000). The empty pIPKTA30N RNAi vector was bombarded two times per experiment as control. The pipetting scheme can be seen in Table 7.

At 24 h post bombardment the number of GFP expressing cells was counted by fluorescence microscopy. Data were normalized to the number of anthocyanin-accumulating cells counted five days post bombardment by light microscopy. These normalized GFP data were expressed as percentage of the mean value of the empty pIPKTA30N RNAi vector.

The current preliminary results based on only one silencing experiment are summarized in Table 11 and Figure 19. The RNAi effect widely varies after this first experiment. There may be silencing effects to the pIPKTA44 construct with many different percentage mlo-RNAi constructs (100%, 90%, 70%, 50%, 10%, and 0%) plus the *Mlo-H1* homologue as reflected by reduced fluorescent signals. For statistical analysis of these preliminary results more repetitions of the experiment will be needed.

Table 11: Data of GFP-fused-*Mlo*-silencing experiments
This Table shows the data of the normalized GFP and the percentage of the relative normalized GFP to the control after one experiment.

Vector	Normalized GFP	% control
pIPKTA30N_Mlo500RNAi-100	0.10	59.0
pIPKTA30N_Mlo500RNAi-90	0.12	73.7
pIPKTA30N_Mlo500RNAi-80	0.17	101.1
pIPKTA30N_Mlo500RNAi-70	0.13	78.9
pIPKTA30N_Mlo500RNAi-60	0.20	118.0
pIPKTA30N_Mlo500RNAi-50	0.10	57.6
pIPKTA30N_Mlo500RNAi-40	No data	
pIPKTA30N_Mlo500RNAi-30	0.16	97.3
pIPKTA30N_Mlo500RNAi-20	0.19	116.4
pIPKTA30N_Mlo500RNAi-10	0.09	51.4
pIPKTA30N_Mlo500RNAi-0	0.12	71.4
pIPKTA30N_Mlo500RNAi-H1	0.13	78.6
average pIPKTA30N	0.17	100.0

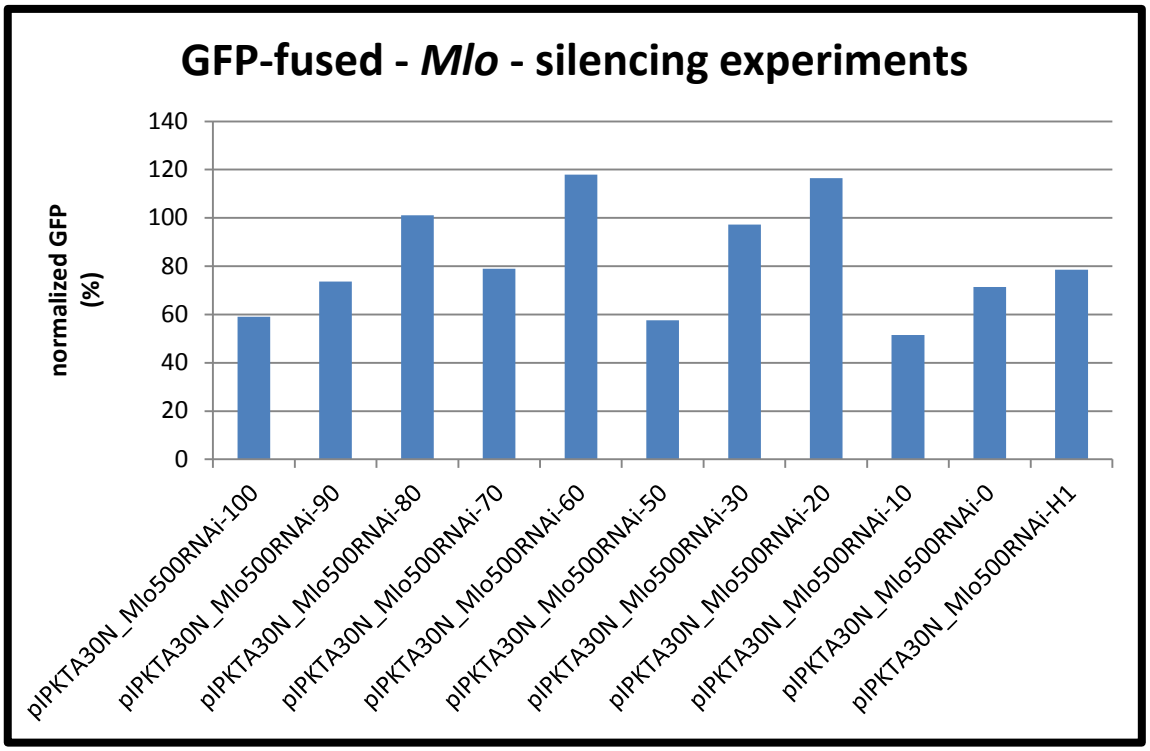

Figure 19: normalized GFP-fused-*Mlo*-silencing experiments
The diagram in this Figure shows the normalized GFP shooting experiments in percent, after one experiment. The pIPKTA30_Mlo500RNAi-40 was removed because no data could be generated in this one experiment.

4 Discussion and outlook

This Bachelor thesis should provide an experimental validation of the "si-Fi" software. Therefore a comparison between *in silicio* prediction of RNAi efficiency and off-target effects in barley with experimental data was performed. The experimental data was generated by two different types of experiments, using a transient gene silencing system in bombarded barley epidermal cells. Thus synthetic RNAi sequences with different similarity from 100% to 0% to the *Mlo* gene and the sequence of the *Mlo* homolog H1 with 80% similarity to *Mlo* were designed and used. First *Mlo*-silencing experiments were done by observing the effect on the resistance to Bgh. Second the GFP-fused-Mlo-silencing experiments were started by analyzing reduction of the fluorescent signal of GFP transcriptionally fused to *Mlo*. However, due to preliminary data of GFP silencing because of missing time to repeat the experiments, discussion of "si-Fi" validation will restricted to the *Mlo*-silencing experiments.
As described in section 3.2 the "si-Fi" prediction suggests that a putative silencing effect of the selected RNAi-trigger sequences can be expected only for the constructs with 100% and partially with the 90% similarity, as well as with the *Mlo-H1* sequence.

The experimental validation of these predictions revealed a strong silencing effect for the 100% and the *Mlo-H1* sequence. In contrast no effect was observed for the 90% similarity construct. It is interesting that the effect from the *Mlo-H1* RNAi sequence is quite stronger than expected relative to the 90% similarity construct.
This shows that the "si-Fi" software is able to produce predictions that approximately fit to the experimental data. Especially, none of the constructs with zero predicted (efficient) siRNAs produced a Bgh resistance phenotype. However, the discrepancy between efficient siRNA prediction and missing Bgh phenotype with the 90% similarity construct suggests that "si-Fi" rather overestimates RNAi efficiency and off-target effects.

The here discussed *Mlo*-silencing experiments were based on observing the effect of the resistance to Bgh thereby only indirectly recording silencing efficiency. This may mean that only upon reaching a certain threshold value of mRNA silencing, the phenotypic effect of reduced amount of MLO protein in the cell, i.e. resistance to Bgh, could be

observed. For this reason, data directly recording target mRNA reduction derived from the GFP-fused-*Mlo*-silencing experiments would be required to refine the analysis.
The random distribution of mutations in the *Mlo* RNAi constructs might have had a major impact on the silencing efficiency. One possibility for testing this assumption experimentally could be comparing a range of randomly generated constructs with 90% similarity to *Mlo*. As an example, the *Mlo-H1* effect may have been initialized by some *bona fide* efficient siRNAs, although only 80% overall sequence similarity to *Mlo* exists.
Another useful experiment for a more sensitive validation of the "si-Fi" software would be a gradual *Mlo* sequence-similarity reduction between 100% and 90%. This could be for example performed by using 98%, 96%, 94%, and 92% *Mlo* similarity constructs

When determining parameters such as the length of siRNA with prediction software like "si-Fi" the target organism should be carefully considered. In mammals it was shown that some 17 bp duplexes even with additional target sequence overhang are not effective, while some other 19 bp siRNAs including four mismatches still can mediate gene silencing (Czauderna et al., 2003; Pancoska et al., 2004). The optimum for effective silencing seems to be siRNA with a precise length of 21 bp, which were generated out of long dsRNA by a RNase III enzyme commonly called Dicer or Dicer-like (DCL) (Elbashir et al., 2001). Between plants siRNAs differ in their length, because there are several different distinct Dicer like (DCL) enzymes. For example *Arabidobsis thalinia* has several different DCL enzymes which produces siRNAs with a length of 21 nucleotides (DCL1 and DCL4), 22 nucleotides (DCL2) and 24-26 nucleotides (DCL3) (Ossowski et al., 2008; Xie et al., 2004). These possible different lengths of siRNA which can occur in plants make the definition of rules, defining parameters for RNAi efficiency and off-target prediction in plants, much more difficult.

As already mentioned above the natural *Mlo-H1* sequence with 80% similarity to *Mlo* showed a much stronger effect than the randomly mutagenized synthetic sequence with 90% similarity. Therefore the percentage similarity to a target sequence seems not to be a highly reliable indicator for efficient silencing. The hybridization of the siRNA with the target mRNA and the RISC complex is a sensitive process, with hybridization

thermodynamics as crucial factors. For example the silencing effect may depend on the position of the siRNA along the target mRNA. Characterized by its secondary structure including loop and stem regions. In addition the nucleotide sequence is an interesting factor. Single nucleotide positions are often essential for binding processes and position-specific mutations can significantly change stability. As it was investigated by (Pancoska et al., 2004) the siRNA efficiency is "based upon the contextual similarity and predicted thermodynamic stability". But especially for plants the factors which influence the activity of the RNAi processes are complex and to a large extent undefined. Thus it seems to be increasingly important to generate *bona-fide* efficient siRNA out of the long dsRNA. The "si-Fi" software currently does not consider these structural aspects determining hybridization conformations. This could explain the false positive prediction for the 90% similarity construct. Regardless to this the amount of siRNA may also have an impact on the silencing effect.

It would be very interesting if further research can test if *Mlo-H1* is expressed or not. And, if it is expressed, which role it might play in the barley-powdery mildew interaction. At least as far as public transcriptional data of barley are concerned *Mlo-H1* appears not or very lowly or specifically expressed. Therefore, it appears more likely that the RNAi effect of *Mlo-H1* was due to off-targeting *Mlo*. The initial sequence of *Mlo-H1* was derived from a genomic Lambda clone of barley (GenBank: Z95496.1), which does not provide clues as to its expression. It appears to be localized to chromosome 4HL based on genetic anchoring of whole-genome shotgun sequences, and might therefore be a tandem duplicate of *Mlo,* which also maps to the same chromosome arm (The International Barley Genome Sequencing Consortium, 2012).
To test whether the protein encoded by *Mlo-H1* really plays a role in the barley-powdery mildew interaction, a transient *Mlo-H1* overexpression experiment using the same experimental system as described here may be informative.

In summary, the "si-Fi" software can be used for off-target prediction, while its usefulness for optimizing RNAi constructs with high efficiency may be limited.

5 Summary

RNAi is an ancient natural antiviral (siRNA) and gene-regulatory (miRNA) mechanism that directs silencing of gene expression in a sequence-specific manner. The silencing of target genes is generally achieved by cutting and degradation of the target mRNA. It can also results from a translational blockade of the intact mRNA by miRNA or from a direct attack on the (promoter) DNA resulting in a transcriptional blockade (Agrawal et al., 2003).

Until now there are no versatile tools for defining parameters for RNAi efficiency and off-target prediction in plants. This Bachelor thesis should provide an experimental validation of the "si-Fi" software, which is designed for RNAi off-target search and silencing efficiency prediction. The aim of this work is a comparison between *in silicio* prediction of RNAi efficiency and off-target effects in plants with experimental data. For this task a transient gene silencing system in bombarded barley epidermal cells and the software "si-Fi", developed by the working group Pathogen-Stress Genomics at IPK, was used.

The experiments were based on using synthetic DNA with different levels of similarity to the original *Mlo* gene of barley that were used as RNAi trigger. The *Mlo* gene is a negative regulator of cell death. Mutation or silencing of the *Mlo* gene confers strong resistance to powdery mildew fungi, which provides an easy observable and quantifiable phenotype or reduced *Mlo* function.

A perfect match to the *Mlo* sequence (100% similarity) and 10 further sequences with decreasing similarity (from 90% to 0% in 10% steps approximately) where designed with a length of 500 bp. In addition a sequence derived from the MLO protein homologue *Mlo-H1* gene of barley sharing approximately 80% similarity to the *Mlo* gene was also included to the experiment as natural sequence with potential off-target effect on *Mlo*. With these designed sequences the *in silico* prediction was performed using software "si-Fi".

The twelve synthetic fragments were cut out of the delivery vector (pUC57) by restriction enzymes *Xba*I and *Sal*I. The excised fragments were inserted to the Gateway™ entry vector pIPKTA38, and transferred by LR recombination reaction to the destination RNAi vector pIPKTA30N.

The efficiency of the different RNAi constructs was estimated by their effect on the resistance of bombarded, transformed epidermal cells to Bgh (Mlo-silencing experiments).

In addition to the approach above, the 100% similarity *Mlo* sequence was transcriptionally fused to an instable GFP reporter, and used as a target of the RNAi constructs. The silencing efficiency of the RNAi constructs was scored by means of reducing the fluorescent signal of GFP (GFP-fused-Mlo-silencing experiments).

The "si-Fi" prediction suggests that a putative silencing effect of the selected RNAi-trigger sequences can be expected only for the constructs with 100% and partially with the 90% similarity to *Mlo*, as well as with the *Mlo-H1* sequence.

Data of the GFP-fused-Mlo-silencing experiments showed principal feasibility of direct off-target testing, but would require further repetitions for a detailed discussion.

The Mlo-silencing experiments showed strong silencing effects for the 100% similarity and the *Mlo-H1* sequences. In contrast no effect was observed for the 90% similarity construct despite off target predictions by "si-Fi". It is noticeable that the effect from the *Mlo-H1* sequence was stronger than for the 90% similarity sequence, although it shared only 80% similarity with *Mlo*. This suggests that the precise sequence and position of off-target siRNAs play a considerable role in RNAi efficiency. In conclusion, "si-Fi" can be used for off-target prediction although it may be rather overestimate off-target effects.

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Appendix

Appendix A: Sequences of synthetic 500 bp *Mlo*

>mlo100

GACGACGAGGCGGACCTCCTCCTGTCGTTAGGATTTAGTCTGTGCACCGGGT
GCGCCACCACAACCGGGTACATGTCCCTAGCCTCCTGCTGGGTCCTTGGCGA
GGTGGGCGCGCTCTGGGGGTCGTCCGACCGCCCCATGCCCTTGTGAAGCAG
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TTCTTCTCCTTGGCCGTGTTCCGCCAGTTGGTGAGCGCCTTGGACGTCTGCTC
GTCGAAGATGGACCTCTTCATGTTTGATCCCATCTGTGTGACGAGCGCGTAG
AGGGGGAAGGTCATATAGCTGCAGAGGAACTGGAGAGCTAGCCCCACCAC
CACCTTCATGATGCTCAGCCCGATCTGCGTGTGGTAGCATTTCTTCAAGCCG
GGCGTGGCCACTGTCCACACAAAATGCGCCATCTG

>mlo90

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CTTCTTCCCCTTGGCCGTGTTCCACCAGTAGGTGAGCGCCTTGGACCACTGC
GCGGCGAAGATGGACCTCTTCCTCTTTGATCCCATCTGTGTGACTAGCGCGT
AGAGGGGACAGGTCATATCGCAGCAGAGACACTGGAGAGCTAGCCCCCCC
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>mlo80

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GTCACGTCGCGTATCATCACATCCCTCAGCATGTCCGGGCCAAGGACTTTCT
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CTCTAAGTTAGACCTATTTATGATTGATGCCAACTGTGTTACGAGCTCGTAG
TGGGCGAAGGTCGTATAGCTGCAGGAGTTCAAGTGACCTGGGCCCAACCCC
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>mlo70
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GCGAAGATGGGCCTATTCATTTATCATTCCATCTTTGTGACTAGCGCGTCAA
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>mlo60
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>mlo50

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>mlo40

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>mlo30

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>mlo20
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>mlo10
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>mlo0
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>MloH1
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GGATGCTGCACGCCGCCGCCTTCCTTCTCGGCCCTCGGGGACGCCGGCACGC
TCTGGGGGTCGTCGGACCGCGCCCCGGCCTTGTGGAGCAGGTGCACCGGCG
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AGCATTTCTTCAAGCCGGGCGTGGCCACTGTCCACACGAAATGAGCCATCT
GAAACGCGTTCTGGAAGAGTGTGAGGTGCATGAGGAA

Appendix B: Pseudocode Mlo shuffle (S. LÜCK and D. DOUCHKOV unpublished)

```
nucleotide_to_modify = sequence_length*percent_wobbeling/100

FOR ALL IN RANGE nucleotide_to_modify:

    nucleotids = ['A', 'T', 'C', 'G']

    pos_to_modify = RANDOM NUMBER sequence_length
    original_nucleotide = nucleotid AT pos_to_modify
    nucleotids = REMOVE original_nucleotide FROM nucleotids
    replacement_nucleotide = RANDOM CHOICE nucleotids
    modified_sequence = REPLACE nucleotid WITH replacement_nucleotide AT pos_to_modify

JOIN modified_sequence
```

Appendix C: Microscopy data *Mlo* - silencing experiments

Vector		1	2	3	4	5	6	7
pIPKTA30N	GUS cells	129	26	339	372	177	60	137
	GUS cells with haustoria	20	5	12	57	27	9	17
pIPKTA30N_Mlo500RNAi-100	GUS cells	60	54	51	454	140	43	103
	GUS cells with haustoria	0	3	2	31	8	3	6
pIPKTA30N_Mlo500RNAi-90	GUS cells	274	332	36	64	41	68	138
	GUS cells with haustoria	7	45	2	8	5	7	12
pIPKTA30N_Mlo500RNAi-80	GUS cells	323	204	275	207	99	184	162
	GUS cells with haustoria	38	16	20	29	8	16	17
pIPKTA30N_Mlo500RNAi-70	GUS cells	201	119	120	323	89	93	126
	GUS cells with haustoria	19	16	14	41	13	12	9
pIPKTA30N_Mlo500RNAi-60	GUS cells	145	200	54	361	60	31	97
	GUS cells with haustoria	11	36	1	36	10	6	7
pIPKTA30N_Mlo500RNAi-50	GUS cells	4	264	177	398	197	156	122
	GUS cells with haustoria	0	24	15	42	27	17	17
pIPKTA30N_Mlo500RNAi-40	GUS cells	352	65	24	273	209	80	130
	GUS cells with haustoria	10	4	4	39	20	9	20
pIPKTA30N	GUS cells	182	67	185	168	313	74	68
	GUS cells with haustoria	16	9	12	18	45	6	10
pIPKTA30N_Mlo500RNAi-30	GUS cells	142	20	10	168	96	151	83
	GUS cells with haustoria	21	6	0	12	15	13	13
pIPKTA30N_Mlo500RNAi-20	GUS cells	211	154	122	240	73	83	138
	GUS cells with haustoria	30	17	5	21	17	11	21
pIPKTA30N_Mlo500RNAi-10	GUS cells	140	111	29	203	161	132	54
	GUS cells with haustoria	9	11	5	20	27	21	5
pIPKTA30N_Mlo500RNAi-0	GUS cells	165	78	99	161	227	138	93
	GUS cells with haustoria	11	5	7	17	28	15	9
pIPKTA30N_Mlo500RNAi-H1	GUS cells	262	235	110	142	110	107	37
	GUS cells with haustoria	11	14	4	12	7	1	1
pIPKTA36	GUS cells	174	206	110	79	194	53	51
	GUS cells with haustoria	3	8	2	5	10	0	4
SNAP34	GUS cells	256	102	73	41	81	95	122
	GUS cells with haustoria	64	23	18	6	21	17	26
pIPKTA30N	GUS cells	81	254	83	150	148	57	118
	GUS cells with haustoria	8	34	6	16	20	10	12

Appendix D: SI indices of *Mlo* - silencing experiments

SI = susceptibility index

Vector	SI	SI	SI	SI	SI	SI	SI
pIPKTA30N_Mlo500RNAi-100	0.000	0.056	0.039	0.068	0.057		0.058
pIPKTA30N_Mlo500RNAi-90	0.026	0.136		0.125		0.103	0.087
pIPKTA30N_Mlo500RNAi-80	0.118	0.078	0.073	0.140	0.081	0.087	0.105
pIPKTA30N_Mlo500RNAi-70	0.095	0.134	0.117	0.127	0.146	0.129	0.071
pIPKTA30N_Mlo500RNAi-60	0.076	0.180	0.019	0.100	0.167		0.072
pIPKTA30N_Mlo500RNAi-50		0.091	0.085	0.106	0.137	0.109	0.139
pIPKTA30N_Mlo500RNAi-40	0.028	0.062		0.143	0.096	0.113	0.154
pIPKTA30N_Mlo500RNAi-30	0.148			0.071	0.156	0.086	0.157
pIPKTA30N_Mlo500RNAi-20	0.142	0.110	0.041	0.088	0.233	0.133	0.152
pIPKTA30N_Mlo500RNAi-10	0.064	0.099		0.099	0.168	0.159	0.093
pIPKTA30N_Mlo500RNAi-0	0.067	0.064	0.071	0.106	0.123	0.109	0.097
pIPKTA30N_Mlo500RNAi-H1	0.042	0.060	0.036	0.085	0.064	0.009	
pIPKTA36	0.017	0.039	0.018	0.063	0.052	0.000	0.078
pIPKTA30N_SNAP34RNAi	0.250	0.225	0.247		0.259	0.179	0.213
average TA30N	0.114	0.134	0.058	0.122	0.144	0.136	0.124

Appendix E: Percentage SI of *Mlo* - silencing experiments

The 13.00 value is placed for the 0 values. The red marked value is an outlier according Nalimov-test.

Vector	%	%	%	%	%	%	%
pIPKTA30N_Mlo500RNAi-100	13.00	41.43	68.18	55.81	39.73		46.87
pIPKTA30N_Mlo500RNAi-90	22.43	101.08		102.17		75.97	69.97
pIPKTA30N_Mlo500RNAi-80	103.29	58.49	126.44	114.51	56.19	64.17	84.44
pIPKTA30N_Mlo500RNAi-70	82.99	100.27	202.84	103.75	101.57	95.22	57.47
pIPKTA30N_Mlo500RNAi-60	66.60	134.24	32.20	81.51	115.89		58.07
pIPKTA30N_Mlo500RNAi-50		67.80	147.34	86.25	95.30	80.42	112.12
pIPKTA30N_Mlo500RNAi-40	24.94	45.89		116.77	66.54	83.02	123.79
pIPKTA30N_Mlo500RNAi-30	129.83			58.38	108.65	63.53	126.03
pIPKTA30N_Mlo500RNAi-20	124.82	82.32	71.25	71.52	161.93	97.80	122.44
pIPKTA30N_Mlo500RNAi-10	56.44	73.90		80.53	116.61	117.41	74.50
pIPKTA30N_Mlo500RNAi-0	58.53	47.80	122.93	86.31	85.77	80.21	77.87
pIPKTA30N_Mlo500RNAi-H1	36.86	44.43	63.22	69.07	44.25	6.90	
pIPKTA36	15.14	28.96	31.61	51.73	35.84	13.00	63.11
pIPKTA30N_SNAP34RNAi	219.48	168.16	428.70		180.27	132.06	171.48

Appendix F: Log2 transformation of *Mlo* - silencing experiments

Vector	Log2	Log2	Log2	Log2	Log2	Log2	Log2
pIPKTA30N_Mlo500RNAi-100	-2.943	-1.271	-0.553	-0.841	-1.332		-1.093
pIPKTA30N_Mlo500RNAi-90	-2.157	0.015		0.031		-0.397	-0.515
pIPKTA30N_Mlo500RNAi-80	0.047	-0.774	0.338	0.195	-0.832	-0.640	-0.244
pIPKTA30N_Mlo500RNAi-70	-0.269	0.004	1.020	0.053	0.022	-0.071	-0.799
pIPKTA30N_Mlo500RNAi-60	-0.586	0.425	-1.635	-0.295	0.213		-0.784
pIPKTA30N_Mlo500RNAi-50		-0.561	0.559	-0.213	-0.069	-0.314	0.165
pIPKTA30N_Mlo500RNAi-40	-2.003	-1.124		0.224	-0.588	-0.268	0.308
pIPKTA30N_Mlo500RNAi-30	0.377			-0.776	0.120	-0.654	0.334
pIPKTA30N_Mlo500RNAi-20	0.320	-0.281	-0.489	-0.484	0.695	-0.032	0.292
pIPKTA30N_Mlo500RNAi-10	-0.825	-0.436		-0.312	0.222	0.231	-0.425
pIPKTA30N_Mlo500RNAi-0	-0.773	-1.065	0.298	-0.212	-0.221	-0.318	-0.361
pIPKTA30N_Mlo500RNAi-H1	-1.440	-1.170	-0.662	-0.534	-1.176	-3.858	
pIPKTA36	-2.724	-1.788	-1.662	-0.951	-1.480	-2.943	-0.664
pIPKTA30N_SNAP34RNAi	1.134	0.750	2.100		0.850	0.401	0.778

Appendix G: Nalimov test for outliers of *Mlo* - silencing experiments

Vector	Nalimov	Nalimov	Nalimov	Nalimov	Nalimov	Nalimov	Nalimov
pIPKTA30N_Mlo500RNAi-100	1.816	0.160	1.399	0.678	0.259		0.157
pIPKTA30N_Mlo500RNAi-90	1.729	0.891		0.928		0.055	0.145
pIPKTA30N_Mlo500RNAi-80	0.626	1.073	1.504	1.051	1.160	0.858	0.089
pIPKTA30N_Mlo500RNAi-70	0.553	0.143	2.290	0.060	0.112	0.263	1.158
pIPKTA30N_Mlo500RNAi-60	0.423	1.507	1.404	0.003	0.984		0.666
pIPKTA30N_Mlo500RNAi-50		1.161	1.876	0.456	0.111	0.679	0.531
pIPKTA30N_Mlo500RNAi-40	1.438	0.857		1.107	0.285	0.172	1.302
pIPKTA30N_Mlo500RNAi-30	1.029			1.230	0.359	1.067	0.909
pIPKTA30N_Mlo500RNAi-20	0.651	0.716	1.072	1.064	1.845	0.218	0.575
pIPKTA30N_Mlo500RNAi-10	1.306	0.549		0.262	1.303	1.337	0.523
pIPKTA30N_Mlo500RNAi-0	0.968	1.453	1.947	0.289	0.265	0.013	0.093
pIPKTA30N_Mlo500RNAi-H1	0.356	0.015	0.937	1.224	0.006	1.825	
pIPKTA36	1.130	0.310	0.153	1.039	0.097	1.256	1.713
pIPKTA30N_SNAP34RNAi	0.028	0.487	2.129		0.366	0.850	0.454

Appendix H: *Mlo* - silencing experiments statistics

Vector	Mean	STD	SEM	T-test (two-sided)	T-test (one-sided)	n
pIPKTA30N_Mlo500RNAi-100	-1.339	0.837	0.342	0.035	0.017	6
pIPKTA30N_Mlo500RNAi-90	-0.604	0.901	0.403	0.570	0.285	5
pIPKTA30N_Mlo500RNAi-80	-0.273	0.482	0.182	0.667	0.334	7
pIPKTA30N_Mlo500RNAi-70	-0.177	0.326	0.133	0.238	0.119	7
pIPKTA30N_Mlo500RNAi-60	-0.444	0.743	0.303	0.781	0.391	6
pIPKTA30N_Mlo500RNAi-50	-0.072	0.393	0.160	0.138	0.069	6
pIPKTA30N_Mlo500RNAi-40	-0.575	0.878	0.358	0.566	0.283	6
pIPKTA30N_Mlo500RNAi-30	-0.120	0.554	0.248	0.397	0.198	5
pIPKTA30N_Mlo500RNAi-20	0.003	0.452	0.171	0.081	0.040	7
pIPKTA30N_Mlo500RNAi-10	-0.258	0.413	0.169	0.589	0.294	6
pIPKTA30N_Mlo500RNAi-0	-0.379	0.436	0.165	0.889	0.445	7
pIPKTA30N_Mlo500RNAi-H1	-1.473	1.217	0.497	0.074	0.037	6
pIPKTA36	-1.745	0.844	0.319	0.005	0.002	7
pIPKTA30N_SNAP34RNAi	1.002	0.587	0.239	0.002	0.001	6

Appendix I: Microscopy data GFP - fused - *Mlo* - silencing experiment

vector	GFP cells	Anthocyanin cells	normalized GFP	% control
pIPKTA30N	45	248	0.181451613	
pIPKTA30N_Mlo500RNAi-100	36	366	0.098360656	59.0198029
pIPKTA30N_Mlo500RNAi-90	34	277	0.122743682	73.6504641
pIPKTA30N_Mlo500RNAi-80	48	285	0.168421053	101.05847
pIPKTA30N_Mlo500RNAi-70	38	289	0.131487889	78.8972914
pIPKTA30N_Mlo500RNAi-60	46	234	0.196581197	117.955532
pIPKTA30N_Mlo500RNAi-50	33	344	0.095930233	57.5614648
pIPKTA30N_Mlo500RNAi-40	0	0		
pIPKTA30N_Mlo500RNAi-30	54	333	0.162162162	97.3029184
pIPKTA30N_Mlo500RNAi-20	39	201	0.194029851	116.424636
pIPKTA30N_Mlo500RNAi-10	33	385	0.085714286	51.4315426
pIPKTA30N_Mlo500RNAi-0	42	353	0.11898017	71.3922262
pIPKTA30N_Mlo500RNAi-H1	30	229	0.131004367	78.6071611
pIPKTA30N	53	349	0.151862464	
Mean pIPKTA30N			0.166657039	100

Statement of authorship

I hereby certify that this bachelor thesis presented here has been composed by myself and is the result of my own investigations, unless otherwise acknowledged in the text. All references and all sources of information have been specifically acknowledged. This thesis has not been submitted, either in part or whole, for a degree at this or any other University. This work has not been published.

Mittweida, 21th August 2013

Tino Kreszies